Università degli Studi di Cagliari

XXVI ciclo di corso di dottorato in Tossicologia

Nematicidal, antimicrobial and acaricidal activity of plant secondary metabolites

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Degree of

European Doctor of Philosophy

Final exam academic year 2012-2013

ACKNOWLEDGEMENTS

Firstly, thanks to God for finalizing this PhD thesis carried out at the Department of Life and Environmental Sciences, Dipartimento di Sanita`Pubblica, Laboratorio di Igiene degli Alimenti, Medicina Clinica e Molecolare at the University of Cagliari, Italy and Instituto di Ciencias Agrarias (ICA-CSIC)-Madrid, Spain, during the years 2011-2014.

I would like to express my sincere gratitude to my Professor Pierluigi Caboni, for giving me the opportunity to peruse a PhD with him. Pierluigi, thank you for the guidance you have giving me over the past of years, providing excellent facilities for my work, enthusiasm, support and mainly for his friendship and sympathy. I have learned so much from you.

I thank Professor Valentina Coroneo for giving me the possibility to work in her laboratory, introducing me to test methods for determining antimicrobial activity of plant extracts as well as giving me her confidence.

My special thanks to Professor Azucena Gonzalez Coloma at Instituto di Ciencias Agrarias (ICA-CSIC)-Madrid, for giving me the possibility to spend a stage period in her laboratory, sympathy and help.

I owe thanks to Professors Alberto Angioni, Carlo Tuberoso and Giorgia Sarais for their help, suggestions for using the analytical instruments, and friendship.

I am grateful to the official reviewers of my thesis, Professors Cedric Bertrand from the University of Perpignan, France and Urania Menkissoglu–Spiroudi from Aristotle University of Thessaloniki, Greece for their valuable comments, suggestions and expert criticism on the manuscript.

I wish to thank all my colleagues for providing a good atmosphere to work, inspiring discussions and for mental support during the creation of this thesis.

My special thanks go to my dear friend Marco Saba for his encouragement and friendship, both at work and during free time.

I am grateful to my dear parents Lamine and Hayet, my sisters Rania and Roeya, my brother Nadim and my uncle Slah for their love, support and confidence during all my academic steps and difficult moments. I would never have got to the end without you. Thank you very much.

My deep and warm thanks are due to my dear Rosy, for her endless love, support and encouragement during all the years of this thesis work. Thank you for everything.

Thanks to my friends Yassin, Dhia and Carlo for their friendship and encouragement.

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INTRODUCTION

It has been estimated that less than 1-10 % of the large diversity of 250.000-500.000 plant species on the Earth have been studied chemically and pharmacologically for their medicinal properties.[1,2] This is especially true for the tropical flora, as at date only 1 % of the species in these habitats have been studied for their pharmaceutical potential.[2] Traditions of collecting, processing and applying plants and plant-based medications, have been handed down from generation to generation.[3] In African countries, traditional medicines, with medicinal plants as their most important components, are sold in marketplaces or prescribed by traditional healers at their homes.[4] Because of this strong dependence on plants as medicines, it would be important to study their safety and efficiency.[5] The value of ethno-medicine and traditional pharmacology is nowadays gaining increasing recognition in modern medicine because the search for new, potential medicinal plants is more successful if the plants are chosen on an ethno-medical basis. It has been estimated that 74% of the pharmacologically active plant-derived components were discovered after the ethno-medical uses of the plants started to be investigated.[6]

Since plants are long-lived stationary organisms, they must resist attackers over their lifetime by producing and exuding secondary metabolites.

In biology, the concept of secondary metabolite can be attributed to Kossel[7]. He was the first to define these metabolites as opposed to primary ones. Thirty years later an important step forward was made by Czapek[8] who dedicated an entire volume of his 'plant biochemistry' series to what he named '*endproduckt*'. According to him, these products could well derive from nitrogen metabolism by what he called 'secondary modifications' such as deamination. Compared to the main molecules found in plants, these secondary metabolites were soon defined by their low abundance, often less than 1% of the total carbon, or a storage usually occurring in dedicated cells or organs. Plant secondary compounds are usually classified according to their biosynthetic pathways.[9] Three large molecule families are generally

considered: phenolics, terpenes and steroids, and alkaloids. A good example of a widespread metabolite family is given by phenolics: because these molecules are involved in lignin synthesis, they are common to all higher plants. However, other compounds such as alkaloids are sparsely distributed in the plant kingdom and are much more specific to defined plant genus and species. This narrower distribution of secondary compounds constitutes the basis for chemotaxonomy and chemical ecology.[10]

Research of phytochemicals has its roots in allelochemistry, involving the chemical-mediated interactions between a plant and other organisms in its environment.[11] Plant secondary metabolites that have no apparent role in processes of plant structure play an important role in UV absorbing, thus preventing serious leaf damage from the light[12] plant-insect interactions,[13] and therefore, such compounds called allelochemicals have nematicidal, insecticidal, hormonal, antifeedant against pests and antimicrobial activities.[14,15]

Nematodes, thread-like worms, mostly about 1 mm in length, are among the most ubiquitous organisms on Earth. One hundred grams of soil typically houses about 3000 individuals.[16] Plant-parasitic nematodes display a wide variety of interactions with their hosts. All have hollow, protrusible stylets, or mouth spears, used to penetrate cells to allow feeding and, for endoparasitic forms, entry into the host. Some nematodes are migratory ectoparasites that never enter the host, but simply migrate through the soil, using roots as an ephemeral food source as they encounter them. Migratory endoparasites enter the host and migrate through host tissues causing extensive damage. Semi-endoparasitic nematodes may have migratory stages, but also partially penetrate the host plant in order to feed at one stage of the life cycle.[17]

Plant-parasitic nematodes called also Root Knot Nematodes (RKN), attack plants and cause roughly US$70 billion of crop losses annually in fruit and vegetable production.[17]

Meloidogyne *spp*. being the most common and widespread group of Root Knot Nematodes in the world[18], increase the severity of soil borne diseases such as Fusarium wilt in watermelon.[19] Plant growth impairment caused by *Meloidogyne* spp. to vegetable crops is influenced by nematode species and physiological race as well as the initial nematode population density in the soil at sowing or transplanting.[20]

Inside the host tissues, Meloidogyne *spp*. pass through an embryonic stage, four juvenile stages (J1−J4), and an adult stage **(Figure 1)**. Juvenile *Meloidogyne* species hatch from eggs as second-stage juveniles (J2), while the first molt occurs within the egg. Newly hatched juveniles live for a short period of time in the rhizosphere of the host plants without feeding. Then J2s invade host root in the root elongation region and migrate until they find a place to settle and feed. In that area, parenchyma cells near the head of the J2 become multinucleate giant cells, from which the J2s and later the adults feed. After further feeding, the J2s undergo morphological changes, and then without further feeding, they molt three times and eventually become adults. In females, the reproductive system develops, and they can produce hundreds of eggs, while male adults leave the root and do not harm the host **(Figures 2, 3)**. The length of the life cycle is temperature-dependent.[21]

It has long been recognized that chemotaxis is the primary means by which nematodes locate host plants. Chemotaxis is a movement in the direction of higher concentrations of semiochemicals such as plant chemical signals. Nematodes J2 are attracted to plant roots via soluble and gaseous attractants produced by the root itself or by attendant rhizosphere micro-organisms.[22,23]

The primary control method for RKN in watermelon, cucumbers, tomatoes, and carrots has been soil fumigation with synthetic nematicides as methyl bromide, metam sodium and 1,3-dichloropropene. In Georgia, RKN significantly reduced fruit yield of ‘Cooperstown’ seedless

watermelon grown in non-treated soil compared to that grown in methyl bromide treated soil.[24] Then, by the means of biological control using bacteria, such us *Bacillus firmus* and *Bacillus chitinosporus*, or using botanical extracts, such as sesame stalk or oil, neem cake, and crab shell meal.[25] The pressure to find viable alternatives to synthetic soil fumigants and methyl bromide, withdrawn in 2005 according to the Montreal Protocol on Substances that deplete the Ozone Layer,[25] and their effect on not-target organisms, has been intensified in recent years. Plant protection from RKN and soilborne plant pathogens should therefore rely on alternative control strategies that are both economically sustainable and environmentally sound at the same time.

Food quality and safety is a scientific discipline describing handling, preparation and storage of food in ways that prevent food borne illness. Food serves as a growth medium for microorganisms that can be pathogenic or cause food spoilage. Therefore, it is imperative to have stringent laws and standards for the preparation, packaging and transportation of food.[26]

There are many types of food borne pathogens that causes food deterioration by affecting the organoleptic characteristics or by toxins production such as *Clostridium perfringens* which produces 17 exotoxins,[27] *E. coli* such as Shiga toxin (STEC) and verocytotoxin-producers (VTEC), including the enterohemorrhagic *E. coli* (EHEC) which constitute one of the most important causes of food-borne disease worldwide.[28] In addition to *Salmonella* spp. and *Staphylococcus aureus* may be found in milk and other dairy products, vegetables, raw and fermented meats, *S. aureus* is regarded as potentially hazardous in foods due to the production of heat-stable enterotoxins (SEs).[29]

Antimicrobial agents have been greatly important for clinical medicine since the second half of the 20th century and was used to treat life-threatening bacterial infections. However, the last decade of the 20th century and the first decade of the 21th century have witnessed the

emergence and spread of antibiotic resistance in pathogenic bacteria around the world, and the consequent failure of antibiotic therapy, especially in intensive care units which has led to hundreds of thousands of deaths annually.[30]

The gradual increase in resistance rates of several important pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus* (VRE), multidrug-resistant (MDR) *Pseudomonas aeruginosa*, imipenem-resistant *Acinetobacter baumannii,* and third-generation cephalosporin-resistant *Escherichia coli* and *Klebsiella pneumonia,* poses a serious threat to public health.[31,32] Therefore, there is an increased public and scientific need to conduct research on the bactericidal efficiency of phytochemicals and other natural substances as viable alternatives to pharmaceutical and chemical antibiotics for medical interest and food preservation.

Listeria monocytogenes is a food-borne pathogen Gram (+) that is emerging as a major public health problem. This pathogen causes food-borne infection and can lead to bacteremia, meningitis, and abortion. *L. monocytogenes* is a highly versatile psychrophilic and facultative aerobic microorganism that is able to survive and proliferate in a wide range of substrates and tolerates acidic environments and high salt concentrations.[33] This bacterium was responsible in the US for 147 illnesses, 33 deaths and miscarriage, due to consumption of cantaloupe in 2011.[34] In addition, a considerable number of produces recalls have occurred in the past three years as a result of *L. monocytogenes* contamination.[35]

Tick infestation and the resulting tick-borne diseases cause serious problems for public and animal health and also have a signficant environmental impact. There are almost 850 tick species and 30 major tick-borne diseases.[36] Species including *Hyalomma lusitanicum* are becoming much more common. These diseases generally affect the blood and/or lymphatic

system and cause symptoms of fever, anaemia, weight loss, milk-drop, lymph node swelling, abortions and death.[36]

Ticks feed several times during their life cycle and can become infected with many pathogens. These can be transmitted to their host-humans and a wide range of animals, including pets and livestock and can cause potentially serious diseases as Crimean-Congo Hemorrhagic Fever (CCHF).

Tropical theileriosis due to *Theileria annulata*, an *Apicomplexan protozoa* transmitted by vector ticks from the genus Hyalomma is a disease affecting cattle throughout a large geographic area covering North Africa, Southern Europe, and Asia where it is extending from the Near East to China.[37] In several developing countries, tropical theileriosis represents a major constraint to the development of cattle industry and production. Infection of cattle with *T. annulata* is the cause of important economic losses.

In order to prevent ticks and the potential diseases they carry, it is important to understand how these pests develop.

The majority of hard ticks require three different hosts to complete their development. During this development, ticks go through four stages of life. These stages are egg, larvae (or seed tick), nymph, and adult. Generally, adult female hard ticks breed while on the host animal and then drop to the ground to lay eggs which will hatch to give larvae with about 1/8-inch in size and have 6 legs. They will use blades of grass and other vegetation to elevate themselves to the height where they can easily grasp onto passing animals such as small rodents or birds. Proximate biochemical signals, such as rising carbon dioxide levels emitted by a warm blooded mammal, alert the ticks to passing hosts.

This procedure is called "questing," and it is used by ticks to find their first host for an initial blood meal. After filling with blood over several days, the seed ticks fall to the ground again, where they molt and become eight-legged nymphs.

The nymph will then lie in wait for a second host to attach to and engorge on blood. Following engorgement, nymphs drop to the ground where they molt again to finally become adult ticks. The adult ticks then go on a hunt for a third, even larger host (ungulate for *Hyalomma*), where they are able to feed and then breed, resulting in reproduction (i.e., eggs). Depending on the species of tick, the entire life cycle can take from two months to years to complete.

Soft ticks differ from hard ticks in that they will develop through several nymph stages, slowly increasing in size until a final molt results in the adult. Their life cycles can take much longer than hard ticks, up to several years in duration. Soft ticks are even known to be able to survive long periods of time without access to a blood meal from a host.

It may be necessary to control tick populations by integrated pest management until we can define the global factors in the growth in tick population.[38] The development of resistance to regular treatments and the potential risk of pathogen transmission are serious obstacles, especially when the current tendency is to use methods compatible with organic farming. Some data have been published about biological tick control using recombinant baculovirus[39], fungi[40] and predators[41]. The use of anti-tick drugs usually includes chemical agents containing synthetic pyrethroids, organophosphates, and amitraz.[42] Although these acaricides are beneficial when properly used, misuse has led to poisoning of humans and animals[43] and serious problems of resistance.

The aims of this thesis were to study the composition of plants extracts belonging to the family of *Simaroubaceae*, *Capparaceae*, *Brassicaceae*, *Meliaceae* and *Fabaceae* and to investigate the activities of their components as aldehydes, ketones, isothiocyanates and polyphenols against root knot nematodes, bacteria and ticks to find potential botanical alternatives to the currently used synthetic pesticides and antimicrobial agents or model compounds for the development of chemically synthesized derivatives with enhanced activity and environmental compatibility and finally to understand and hypothesize the mode of action of these secondary metabolites.

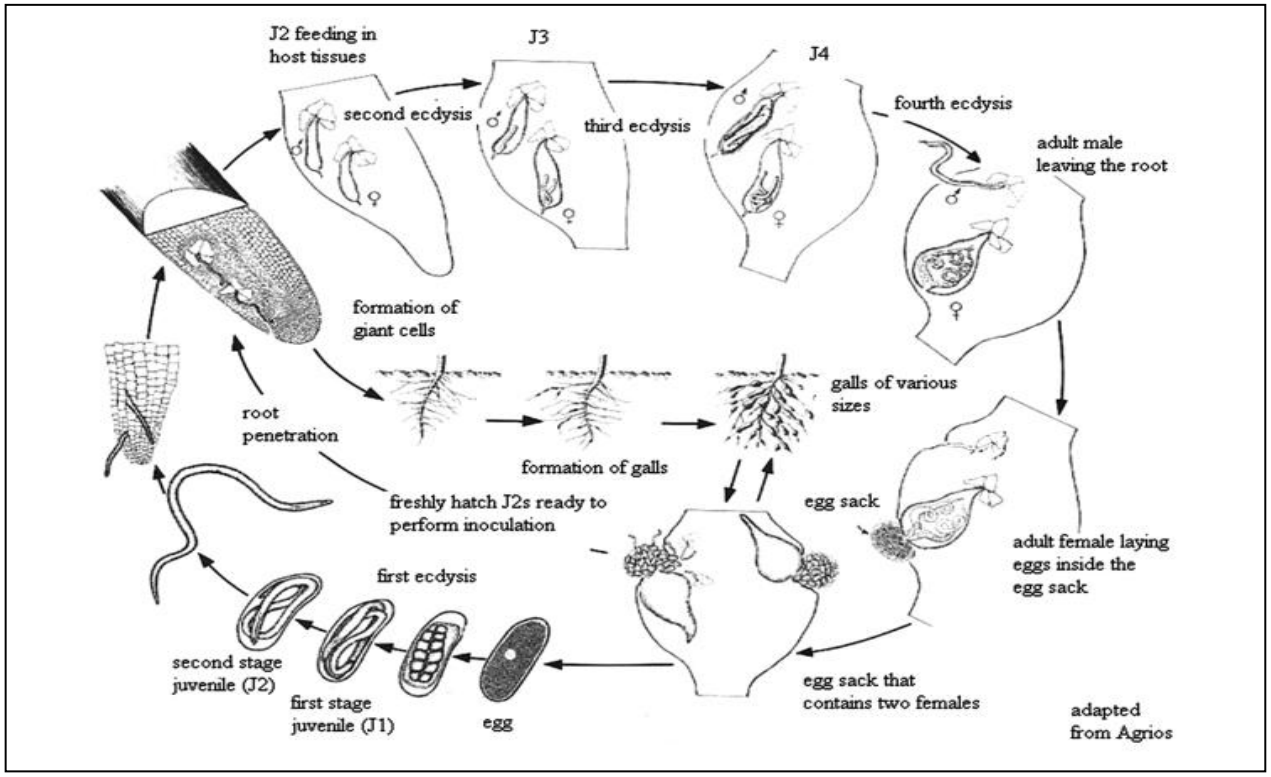


Figure 1: Biological cycle of *Meloidogyne* spp.

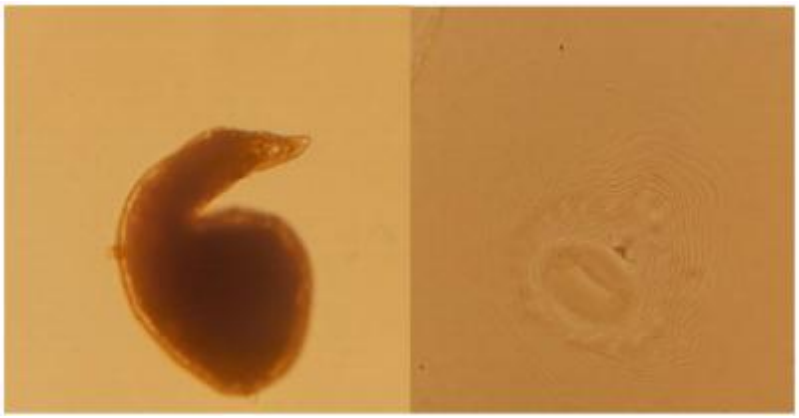


Figure 2: Female of *Meloidogyne incognita* (left) and perineal pattern (right).

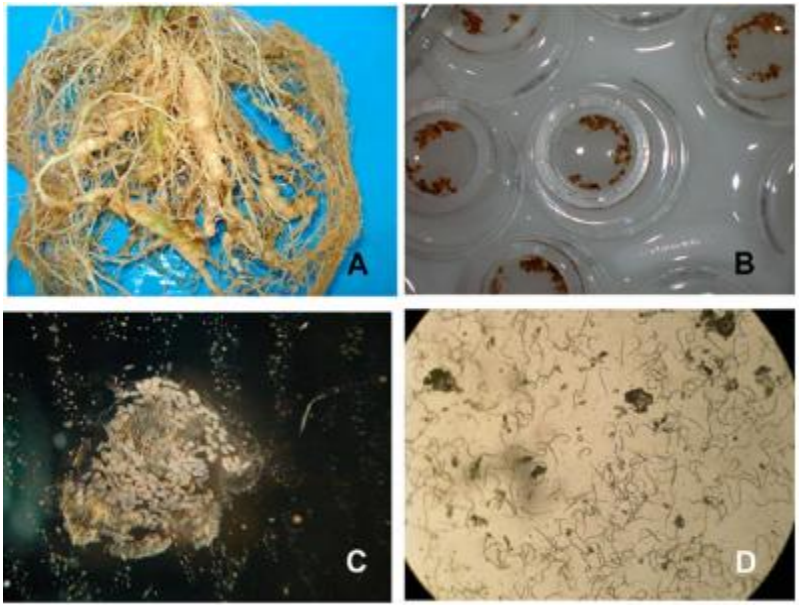


Figure 3: *Meloidogyne incognita* infested tomato root (A) with batches of egg masses for egg hatch (B), eggs inside egg mass (C), and hatched juveniles (D).

CHAPTER I

NEMATICIDAL ACTIVITIES OF ALDEHYDES, KETONES, ISOTHIOCYANATES AND POLYPHENOLS AGAINST *MELOIDOGYNE* SPP.

I. Nematicidal Activity of (E,E)-2,4-decadienal and (E)-2-decenal from *Ailanthus altissima* against *Meloidogyne javanica*:

I. 1. *Ailanthus altissima*:

Ailanthus altissima, commonly known as "tree of heaven" is a deciduous tree of the *Simaroubaceae* family. *A. altissima* is native to northeast and central China and was introduced in Europe as a street tree at the end of the 18th century. *A. altissima* grows rapidly and is capable of reaching heights of 10-15 m, and for this reason, it has become invasive specie capable of colonizing disturbed areas **(Figure 4)**. When the leaves and flowers are crushed, they emit a foul-smelling odour. The characteristics of this plant include the versatility of the reproductive methods, the tolerance to unfavourable conditions, and the potential presence of allelochemicals.[44] The tree of heaven has already been used in traditional medicine in many parts of Asia, including China, while the bark and leaves are being used for their bitter-tonic, astringent, vermifuge, and antitumoral properties.[45] Different phytochemical studies reported the presence in the plant of chemical compounds, such as quassinoids, alkaloids, lipids and fatty acids, volatile and phenolic compounds, flavonoids, and coumarins.[45] Kraus et al. reported that the ailanthone extracted with methanol from *A. altissima* seeds turned out to be a potent antifeedant and insect growth regulator.[46]

I. 2. Materials and Methods:

I. 2. a. Chemicals:

Standards of (E,E)-2,4-decadienal, (E)-2-undecenal, (E)-2-decenal, (E)-2-octenal, nonanal, hexanal, acetic acid, furfural, 2,3-butanediol, hexanoic acid, 5-hydroxymethylfurfural, heptanal, of purity greater than 98%, Tween 20, and dimethylsulfoxide were obtained from Sigma-Aldrich (Milano, Italy). Methanol and water were high-performance liquid chromatography (HPLC)-grade.

Figure 4: *Ailanthus altissima* tree

I. 2. b. Plant Materials:

Leaves, bark, and wood of *A. altissima* were collected before flowering in April 2011 at Cagliari, Italy, and were dried in the absence of light at room temperature. Then, they were sealed in paper bags, stored at room temperature, and kept in the dark until use. Voucher specimens were deposited at the Department of Pharmaceutical Chemistry and Technology, University of Cagliari, Cagliari, Italy, for species identification.

I. 2. c. *A. altissima* Methanol Extracts:

Dried leaves, bark, roots, and wood plant parts (100 g) were ground and extracted with methanol (1:10, w/v) in a sonicator apparatus for 15 min, filtered through a Whatman no. 40 filter paper, and centrifuged for 15 min at 13 000 rpm. The extract was analyzed for component identification by the mean of GC-MS.

I. 2. d. GC-MS Analysis:

The chromatographic separation and identification of the main components of methanol extracts of *A. altissima* were performed on a Trace GC ultra gas chromatograph (Thermo

Finnigan, San Jose, CA) coupled with a Trace DSQ mass spectrometry detector, a split−splitless injector, and an Xcalibur MS platform. The column was a CP-WAX 57CB from Varian (60 m, 0.25 mm inner diameter, and 0.25 μ m film thickness; Varian, Inc., Palo Alto, CA). The injector and transfer line were at 200 °C. The oven temperature was programmed as follows: 50 ° C (held for 1 min), then raised to 220 °C (3 ° C/min), and isothermally held for 13 min. Helium was the carrier gas at a constant flow rate of 1 mL/min; 1 μL of each sample was injected in the splitless mode (60 s). Mass spectrometry acquisition was carried out using the continuous [electron ionization (EI) positive] scanning mode from 40 to 500 amu. *A. altissima* methanol extract components were identified by (1) comparison of their relative retention times and mass fragmentation to those of authentic standards and (2) computer matching against a NIST98 commercial library, National Institute of Standards and Technology (NIST), Gaithersburg, MD. Quantitative analysis of each component was carried out with the external standard method.

I. 2. e. Effect of *A. altissima* Extracts on J2 Motility:

For the assessment of the effect of all extracts used in this thesis against J2 of *Meloidogyne* spp., the method below was used. In the present case, effects of *A. altissima* wood extract (AWE), *A. altissima* leaves extract (ALE), *A. altissima* bark extract (ABE), and *A. altissima* roots extract (ARE) on *M. javanica* J2 motility were tested at the test concentration range of 15.6−250 mg/L for EC50 calculation. Pure compounds contained in the extracts were tested individually against *M. javanica* at the concentration range of 1−50 mg/L for EC50 calculation. The compounds used for the paralysis experiment were (E,E)-2,4-decadienal, (E)-2-decenal, (E)-2-undecenal, (E)-2-decenal, nonanal, heptanal, hexanal, furfural, and 5-hydroxymethylfurfural. Stock solutions were prepared in methanol to overcome insolubility, whereas Tween 20 in distilled water was used for further dilutions. Final concentrations of methanol in each well never exceeded 1% (v/v) because preliminary

experiments showed that this concentration was not toxic to nematodes. Distilled water as well as a mixture of water and Tween (0.3%, v/v) served as controls. In all cases, working solutions were prepared containing double the test concentration and mixed in CellstarR 96-well cell culture plates (Greiner Bio-One) at a ratio of 1:1 (v/v) with suspensions of 15 J2 added to each well. Multi-well plates were covered to prevent evaporation and maintained in the dark at 28 °C. Juveniles were obtained by an Italian population of *M. javanica* reared for 2 months on tomato (*Solanum lycopersicum*) in a glasshouse at 25±2 °C and isolated using Bearman funnels. Briefly, roots are cut and placed in a funnel of 72 µm of diameter in contact with water which is the reagent of natural hatch of eggs from egg sacs. In this manner, eggs will hatch and J2 will pass throughout the funnel to water. After 2 days of incubation in dark and 27 °C, J2 are recuperated filtrating the water using a funnel of 38 µm of diameter. Juveniles were observed with the aid of an inverted microscope (Zeiss, 3951, Germany) at 10×after 1, 24, and 72 h and were ranked in to two distinct categories: motile or paralyzed. Moreover, at that point, nematodes were moved to plain water after washing in tap water through a 20 µm pore screen to remove the excess of extracts. Numbers of motile and paralyzed J2 were assessed by pricking the juvenile body with a needle, and they were counted.

I. 2. f. Statistical Analysis:

Treatments of paralysis experiments were replicated 5 times, and each experiment was performed twice. The percentages of paralyzed J2 in the microwell assays were corrected by elimination of the natural death/paralysis in the water control according to the Schneider Orelli formula:[47]

Corrected percent = [(mortality percent in treatment−mortality percent in control)/(100−mortality percent in control)] × 100, and they were analyzed by analysis of variation

(ANOVA) combined over time. Because ANOVA indicated no significant treatment by time interaction, means were averaged over experiments. Corrected percentages of paralyzed J2 treated with *A. altissima* extracts or pure compounds were subjected to non linear regression analysis using the log−logistic equation proposed by Seefeldt et al.:[48]

$Y = C + (D - C)/\{1 + \exp[b(\log(x) - \log(EC50))]\}$, where C is the lower limit, D is the upper limit, b is the slope at EC50, and EC50 is the *A. altissima* extract or pure compound concentration required for 50% death/paralysis of nematodes after elimination of the control (natural death/paralysis). In the regression equation, the *A. altissima* extract or pure compound concentration (%, w/v) w as the independent variable (x) and the paralyzed J2 (percentage increase over water control) was the dependent variable (y). The mean value of the five replicates per test concentration and immersion period was used to calculate the EC50 value.

I. 2. g. Scanning Electronic Microscopy (SEM) Analysis:

The physical mechanism that (E,E)-2,4-decacadienal and furfural used to interact with the external nematode cuticle was observed by SEM in the environmental mode (1−20 Torr). Freshly hatched nematodes were treated for 24 h by immersion in a 100 µL solution containing 100 mg/L test compounds. Thereafter, a topographic visualization using a FEI Quanta 200 microscope (FEI, Hillsboro, OR) was performed.

I. 3. Results:

Using a CP-WAX 57CB Carbowax column, we were able to separate polar and medium polar plant secondary metabolites, such as (E,E)-2,4-decadienal, (E)-2-undecenal, (E) 2-decenal, (E)-2-decenal, hexanal, nonanal, acetic acid, furfural, 2,3-butanediol, hexanoic acid, and 5-hydroxymethylfurfural **(Table 1)**. Among unsaturated aldehydes identified in *A. altissima* extracts, (E,E)-2,4-decadienal, (E)-2-decenal, and furfural were the most active against J2 with EC50/1d of 11.70, 20.43 and 21,79 mg/L, respectively, while (E)-2-octenal, nonanal,

heptanal, and hexanal did not provoke paralysis on J2 **(Table 2)**. Interestingly, 1 h post J2 immersion in test solutions, EC50 values were calculated even lower [7.5 and 11.75 mg/L for (E,E)-2,4-decadienal and (E)-2-decenal], but this activity was characterized as nematostatic rather than nematicidal because to some extend J2 regained their movement later. Moreover, no fumigant activity of plant methanol extracts or pure compounds was detected. The activity of (E,E)-2,4-decadienal and (E)-2-decenal against *M. javanica* is rather high if compared to the nematicidal activity of fosthiazate (EC50/1d= 15.9 mg/L). According to the GC-MS analysis **(Figure 5)**, AWE afforded (E,E)-2,4-decadienal, (E)-2-decenal, hexanal, nonanal, acetic acid, furfural, 2,3-butanediol, 2-decenal, 2-undecenal, hexanoic acid, and 5-hydroxymethylfurfural**,** while ALE, ARE, and ABE afforded nonanal, acetic and hexanoic acids, and 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one. As a result of the GC-MS analysis, 14 plant metabolites, accounting for 82.6% of the methanol extract, were identified. Without taking into account plant compound bioavailability or the synergetic effect when AWE was tested against *M. javanica*, a clear dose response relationship was established and significant paralysis of J2 was evident after 3 days of exposure, with an EC 50/3d value calculated at 58.9 mg/L **(Table 3)**. This is the first report of the irreversible nematicidal activity of unsaturated aldehydes as constituents of *A. altissima* against *M. javanica*. According to our results, (E,E)-2,4-decadienal and (E)-2-decenal were the principal nematicidal constituents of AWE. Interestingly, the other aldehydes or ketones were not found nematicidal against RKN. Our results clearly indicate that α, β, γ, δ -unsaturated C10 aldehydes are generally more potent nematicidal than their shortest C chain counterparts versus *M. javanica* in vitro experiments. Kim et al. reported that α, β-unsaturated aldehyde 2-decenal showed the highest nematicidal activity at 200 mg/L against the pine wood nematode (*Bursaphelenchus xylophilus*) if compared to other non-unsaturated C8−C10 aldehydes.[49] On the other hand, Andersen et al. reported that C9 unsaturated aldehydes, i.e.,

(E)-2-nonenal and (E,Z)-nonadienal, showed the strongest antifungal activity against *Alternaria alternata* if compared to shortest chain aldehydes, concluding that the effectiveness is due to their increased propensity to react with thiols and amino groups of the target fungi.[50]

Understanding the mode of action of the α, β and α, β, γ, δ unsaturated C10 aldehydes is of practical importance for developing new formulations and delivery systems for nematode control. We observed that nematodes treated with aldehydes and ketones were paralyzed in a straight shape, in a similar way as reported by Kim et al. that treated nematodes with plant essential oils,[49] while Kong et al. reported that pine wood nematode treated with muscle activity blockers levamisole or morantal tartrate were paralyzed in semi-circular and coiling shapes, respectively **(Figure 6)**.[51] Moreover, we have recently reported the circular shape paralysis of J2 after immersion with the organoposphorous fosthiazate.[52] Aliphatic aldehydes and to a lesser extent ketones are relatively reactive compounds. The carbonyl carbon is an electrophilic site and reacts with primary amines and thiols, resulting in the formation of substituted imines, called Schiff bases and hemiacetals, respectively. Aldehydes bringing one or two insaturations become even more reactive, being easily the site of nucleophilic attack. Taking into account the reactivity of α, β, γ, δ unsaturated aldehydes and environmental scanning electron microscopy (ESEM) experimental photographs of the external nematode cuticle following treatment with (E,E)-2,4-decadienal and furfural at 100 mg/L led us to hypothesize the reaction of α, β, γ, δ aldehydes with the nematode cuticle through a Michael addition. This reaction consists of a nucleophilic addition of a cuticle amino or thiol group to an α, β-unsaturated carbonyl. This interaction leads to evident cuticle damage and leakage of the internal fluid nematode material **(Figure 7)**. Similar nematode cuticle damages were reported for *Panagrellus redivivus* caused by a unique fungal structure on the vegetative hyphae of *Coprinus comatus*. The latter was also able to produce potent nematicidal toxins,

such as 5-methylfuran-3-carboxylic acid and 5-hydroxy-3,5-dimethyl-furan-2(5H)-one.[53,54] Moreover, Luo et al. observed that the fungus *Stropharia rugosoannulata* produced a severe mechanical damage on the cuticle of nematodes *Panagrellus redivivus* and *Bursaphelenchus xylophilus* through finger-like projections called acanthocytes.[55]

Table 1: Composition of AWE, ALE, ABE and ARE determined by GC-MS analysis and listed in order of elution:

compound	mol. wt.	t_R (min)	EIMS *m/z* (amu), (abundance)	mol. wt.	AWE (mg/kg)	ALE (mg/kg)	ABE (mg/kg)	ARE (mg/kg)
hexanal		10.88	56 (100%); 72(63%); 82(47%)	100.2	179	-	-	-
nonanal		22.71	57 (100%); 82 (51%); 70 (49%)	142.2	61.1	-	76	-
(E)-2-octenal		24.27	70 (100%); 55 (82%); 83 (70%)	126.1	44.7	-	-	-
acetic acid		25.59	60 (100%); 69 (10%)	60.0	108	271	419	280
furfural		25.84	95 (100%); 94 (91%); 59 (13%)	96.1	1.38	-	-	-
[R-(R*,R*)]-2,3-butanediol		28.73	60 (100%); 75 (80%)	90.1	7.36	-	-	-
[S-(R*,R*)]-2,3-butanediol		30.20	57 (100%); 75 (60%); 72 (20%)	90.1	14.7	-	-	-
2-decenal		32.89	70 (100%); 55 (77%); 83 (65%)	154.2	33.2	-	-	-
2-undecenal		36.99	70 (100%); 83 (73%); 55 (62%)	168.3	37.7	-	-	-
(E,Z)-2,4-decadienal		37.52	81 (100%); 83 (28%); 67 (17%)	152.2	68.6	-	-	-
(E,E)-2,4-decadienal		39.20	81 (100%); 83 (19%); 67 (18%)	152.2	124	-	-	-
hexanoic acid		40.55	56 (100%); 73 (79%); 87 (24%)	116.2	48	4	11.88	13.3
2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one		54.52	97 (100%); 126 (79%); 69 (31%)	144.1	NC	NC	NC	NC
5-hydroxymethylfurfural		61.59	144 (100%); 101 (48%);73 (28%)	126.1	70	-	-	-

Table 2: EC50 and R^2 values of individual compounds against *M. javanica* calculated at 1h and 1 day after immersion in test solutions:

compound	1 h		1 day	
	EC_{50} (mg/L)	R^2	EC_{50} (mg/L)	R^2
(*E*,*E*)-2,4-decadienal	7.53	0.98	11.70	0.97
(*E*)-2-decenal	11.75	0.98	20.43	0.93
(*E*)-2-undecenal	>25		>25	
(*E*)-2-octenal	na[b]		>25	
nonanal	>50		na	
heptanal	na		na	
hexanal	na		na	
furfural	>25		21.79	0.98
5-hydroxy-methylfurfural	>25		>25	
fosthiazate	>25		15.9	0.98

[a]If R^2 values are not presented and the EC_{50} values have not been calculated, they were outside the test concentration range and are estimated higher than the upper concentration level (25 mg/L). [b]na = not active in the range of 25−1000 mg/L.

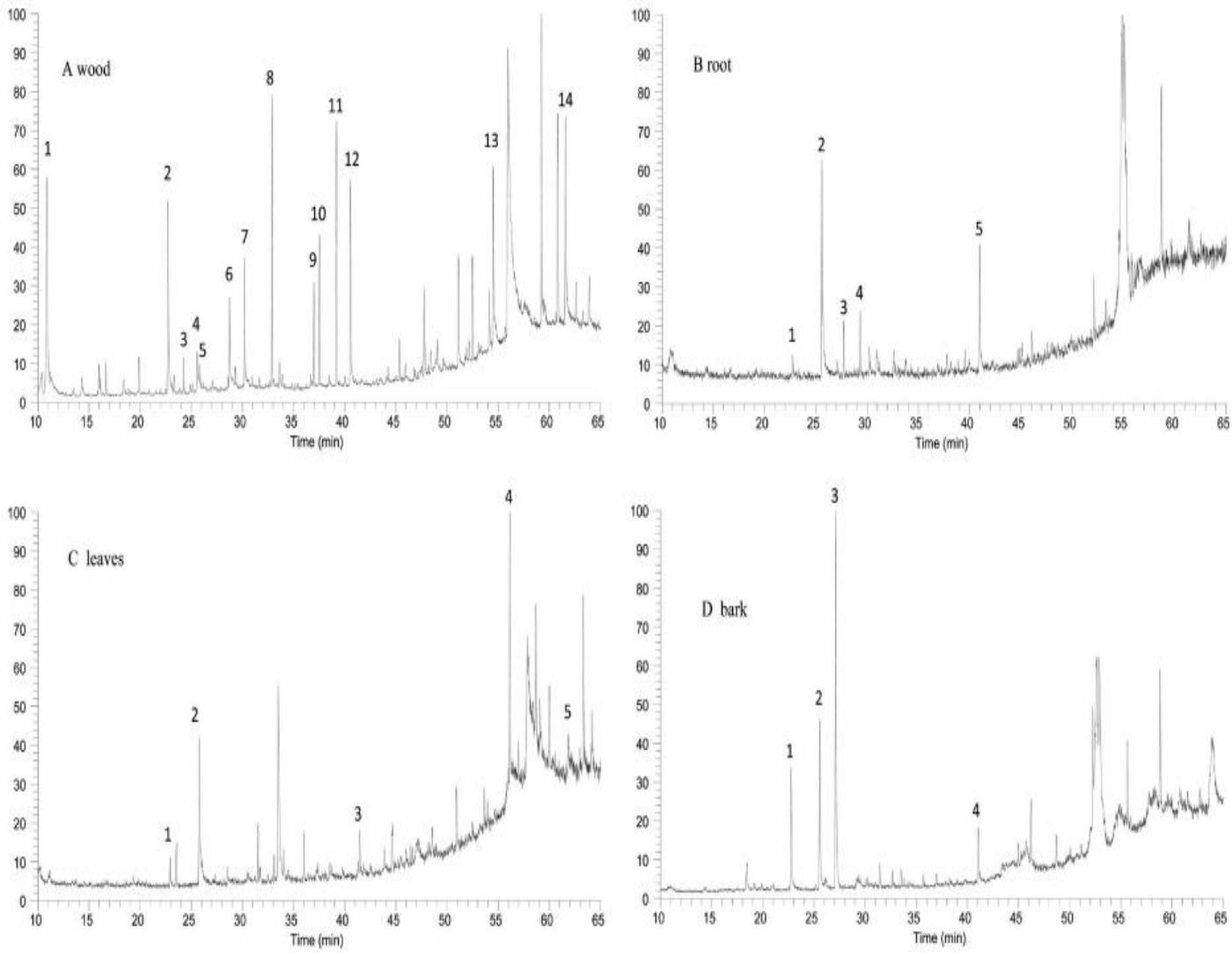


Figure 5: GC-MS chromatogramms of *A. altissima* methanol extracts. Peaks A: (1) hexanal, (2) nonanal, (3) (*E*)-2-octenal, (4) acetic acid, (5) furfural, (6) (*E,Z*)-2,3-butanediol, (7) (*E,E*)-2,3-butanediol, (8) (*E*)-2-decenal, (9) (*E*)-2-undecenal, (10) (*E,Z*)-2,4-decedienal, (11) (*E,E*)-2,4-decedienal, (12) hexanoic acid, (13) 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one, and (14) 5-hydroxymethylfurfural. Peaks B: (1) nonanal, (2) acetic acid, (3) 2-nonanol, (4) 1-octanol, and (5) hexanoic acid. Peaks C: (1) nonanal, (2) acetic acid, (3) hexanoic acid, (4) 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one, and (5) 5-hydroxymethylfurfural. Peaks D: (1) nonanal, (2) acetic acid, (3) decanal, and (4) hexanoic acid.

Table 3: EC50 and R^2 Values of *A. altissima* Extracts (ALE, ABE, AWE, and ARE) against *M. javanica* Calculated after 3 Days of Immersion in Test Solutions and Respective Concentration of Each Extract Provoking 100% Paralysis:

	3 days		4 days	
extract	EC_{50} (mg/L)	R^2	100% mortality (mg/L)	100% mortality (mg/L)
AWE	58.9	0.91	500	31.2
ABE	>250		625	312
ALE	>250		>2500	>2500
ARE	>250		>2500	>2500

[a]*A. altissima* methanolic extracts of wood, AWE; leaves, ALE; bark, ABE; and roots, ARE.

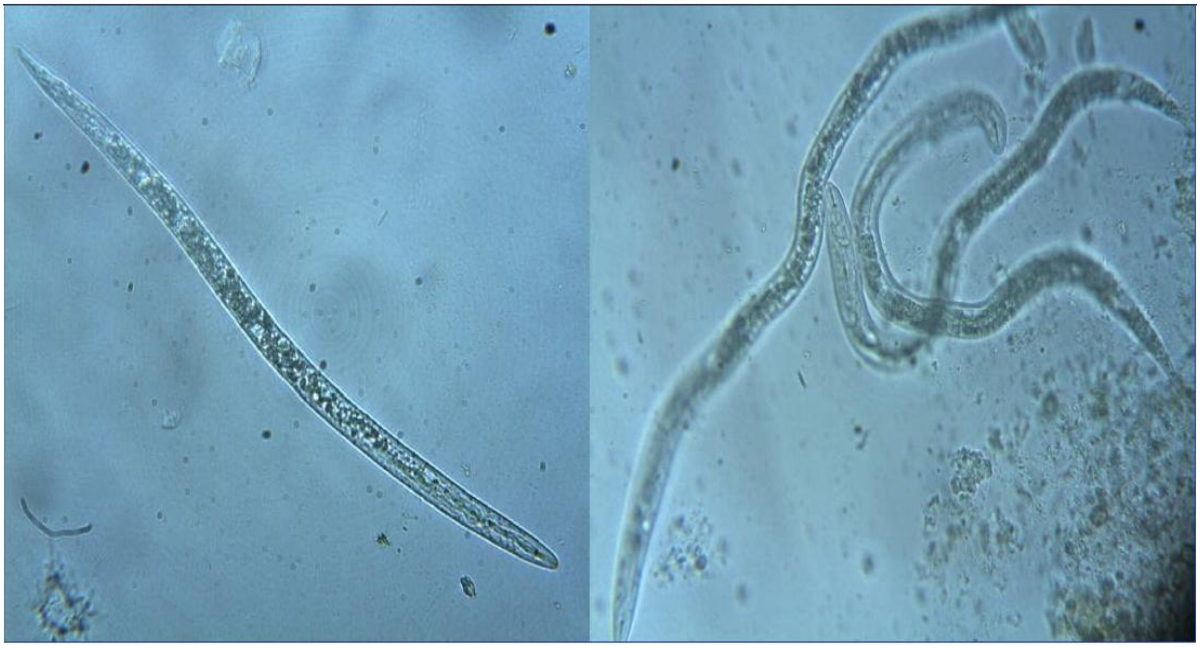


Figure 6: Nematodes paralyzed in a straight shape (left), semi-circular and coiling shapes (right)

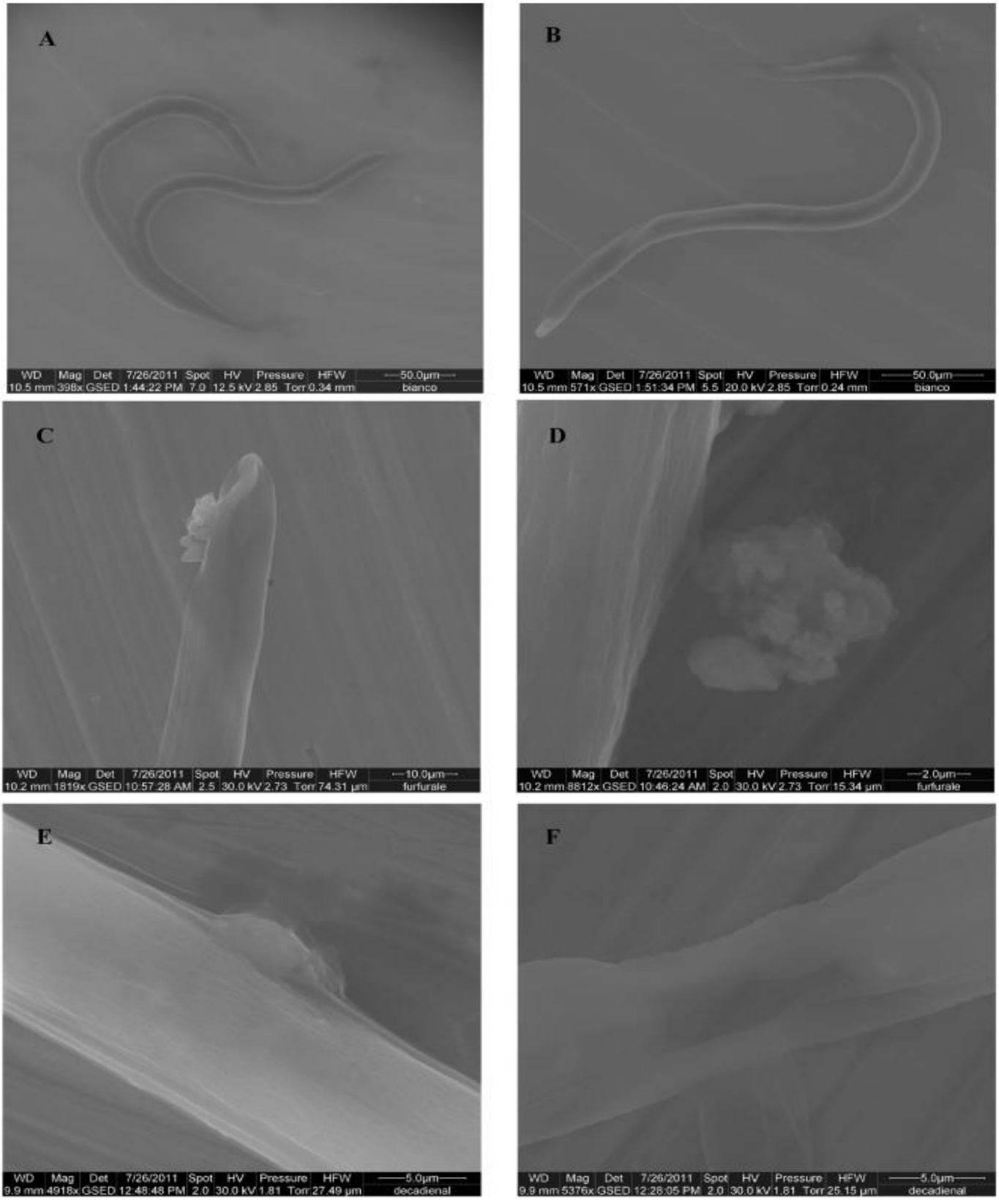


Figure 7: ESEM topographical images from immersion treatment of J2 with (A and B) water, (C and D) furfural at 100 mg/L, and (E and F) treatment with (*E*,*E*)-2,4-decadienal at 100 mg/L. (D−F) Degradation of the external cuticle of *M. incognita* juveniles is evidenced by the leakage ofinternal fluids.

II. Potent Nematicidal Activity of Phthalaldehyde, Salicylaldehyde, and Cinnamic Aldehyde against *Meloidogyne incognita*:

II. 1. Materials and Methods:

II. 1. a. Chemicals:

All of the chemicals were of purity > 98%. Phthalaldehyde **(1)**, salicylaldehyde **(2)**, cinnamic aldehyde **(3)**, 2-naphthaldehyde **(4)**, piperonal **(7)**, 3-hydroxybenzaldehyde **(6)**, citral **(9)**, pyocyanin **(10)**, 4-hydroxybenzaldehyde **(11)**, 2-nitrobenzaldehyde **(12)**, phenylacetaldehyde **(13)**, benzaldehyde **(14)**, cinnamic acid **(15)**, helicin **(16)**, 3,4-methylenedioxyacetophenone **(17)**, catechol **(18)**, benzoic acid-2-hydroxymethylester **(19)**, 3-formylbenzoic acid **(20)**, 2-methoxybenzaldehyde **(21)**, 3,4-dihydroxybenzaldehyde **(22)**, 3,4-dimethoxybenzaldehyde, vanillin **(23)**, furfural **(24)**, abamectin, levamisole, ouabain, (E,E)-2,4-decadienal, and fosthiazate as well as Tween 20 and dimethyl sulfoxide were obtained from Sigma-Aldrich (Milano, Italy). Isophthalic dicarboxaldehyde **(5)** was obtained from Alfa Aesar. Methanol and water were of HPLC grade. The peptide N-acetyl-EEQCTSCVQLQCP (88% of purity) was purchased from Genscript USA Inc.

II. 1. b. General Procedure for the Formylation of Phenols I and III.[56]

A solution of the appropriate phenol (4.09 mmol) in 2.5 mL of 10 N NaOH was heated to 65 °C. After that, 1 mL of $CHCl_3$ was slowly added to the mixture, which was then heated at reflux in chloroform for 2 h. After cooling, the mixture was acidified to pH 1 with concentrated HCl, the organic layer collected, and the aqueous phase extracted with $CHCl_3$. The combined organic phase, previously dried on Na_2SO_4, was concentrated to give a crude product, which was purified by flash chromatography on silica gel **(Figure 8)**.

2-Hydroxy-4-pentadecylbenzaldehyde: ^{1}H NMR (500 MHz,CDCl3) δ 9.95 (s, 1H), 7.62 (s, 1H), 6.89 (d, J_3= 9 Hz, 1H), 6.56 (d,J_3= 9 Hz, 1H), 1.55 (m, 28H), 1.16 (t, 3H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 191.2, 162.3, 145.1, 134.8, 121.2, 36.7, 30.2, 16.9.

5-Ethyl-2-hydroxybenzaldehyde **(8)**: ^{1}H NMR (500 MHz, CDCl3) δ 10.0 (s, 1H), 7.52 (s, 1H), 7.21 (d, J_3= 9 Hz, 1H), 6.97 (d, J_3= 9 Hz,^{1}H), 2.65 (q, J_3= 11 Hz, 2H), 1.32 (t, J_3= 11 Hz, ^{3}H); ^{13}C NMR (500 MHz, CDCl3) δ 189.1, 172.0, 163.5, 141.0, 122.4, 112.3, 33.5,17.7.

II. 1. c. General Procedure for Aldehyde Immobilization on PEG.

All PEG samples (Aldrich) were melted under vacuum at 90 °C for about 45 min before use to remove any trace of moisture. After reaction, the crude mixture was concentrated under vacuum to eliminate the solvent, and then 6-7mL of CH_2Cl_2 was added to completely dissolve the residue. The obtained mixture was added to Et_2O (50 mL/g polymer) cooled at 0 °C. The obtained suspension was filtered through a sintered glass filter, and the solid obtained was repeatedly washed on the filter with pure Et_2O. All of the samples have been crystallized from isopropyl alcohol, to eventually eliminate the excess of the polar reagents or the by products. The yields of PEG-supported compounds were determined by weight. The indicated yields were for pure products after crystallization from isopropyl alcohol. Their purity was confirmed by 500 MHz ^{1}H NMR analysis in $CDCl_3$ with a presaturation of the methylene signals of the polymeric support at 3.60 ppm. When the NMR spectra were recorded, a relaxation delay of 6 s and an acquisition time of 4 s were used to ensure complete relaxation and accuracy of integration. The integrals of the signals of the PEG CH_2OCH_3 fragment at δ 3.30 and 3.36 were used as internal standards.

4-OPEG-benzaldehyde. The synthetic procedure for the preparation of PEG polymer-bound aldehyde started by mesylating the monomethyl ether of polyethylene glycol **(V)** with M_w = 5000 Da. Then, the 4-hydroxybenzaldehyde **(11)** was anchored to PEG-mesylate 2 **(VI)**, giving final product VII **(Figure 9)**.[57]

PEG monomesylate **(V)**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.08 (s, 3H).

4-OPEG-benzaldehyde **(VII)**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.87 (s, 1H), 7.82 (d, J_3= 7 Hz, 2H), 7.015 (d, J_3= 7 Hz, 2H).

II. 1. d. Effect of Different Aromatic Aldehydes on J2 Motility.

The solutions of the aromatic aldehydes were tested on J2 motility of *M. incognita* at different concentration ranges for EC_{100s} at 1h and EC_{50s} at 1 day of calculation as described before. The compounds used for the paralysis experiment were phthalaldehyde **(1)**, salicylaldehyde **(2)**, cinnamic aldehyde, 2-naphthaldehyde **(4)**, piperonal **(7)**, 3-hydroxybenzalde-hyde **(6)**, citral **(9)**, pyocyanin **(10)**, 4-hydroxybenzaldehyde **(11)**, 2-nitrobenzaldehyde **(12)**, phenylacetaldehyde **(13)**, benzaldehyde **(14)**, cinnamic acid **(15)**, helicin **(16)**, 3,4-methylendioxyacetophenone **(17)**, catechol **(18)**, benzoic acid-2-hydroxy methyl ester **(19)**, 3-formylbenzoic acid **(20)**, 2-methoxybenzaldehyde **(21)**, 3,4-dihydrox-ybenzaldehyde **(22)**, vanillin **(23)**, furfural **(24)**, abamectin, levamisole, ouabain, (E, E)-2,4- decadienal, fosthiazate, isophthalic dicarboxaldehyde **(5)**, 5-ethyl-2-hydroxybenzaldehyde **(8)**, 2-hydroxy-4-pentadecylbenzaldehyde, 3,4-dimethoxybenzaldehyde, and 4-OPEG benzaldehyde.

II. 1. e. SEM Characterization.

Freshly hatched J2 nematodes were treated for 24 h by immersion in two 100 µL solutions containing respectively 100 mg/L of salicylaldehyde **(2)** and 100 mg/L of

(E,E)-2,4-decadienal. Thereafter, both treated and untreated nematodes, the latter used as negative controls, were post fixed in a solution of osmium tetroxide (1%) in 0.1 M cacodylate buffer for 1 h and extensively washed in distilled water. The specimens were then rapidly frozen in slush nitrogen (SN_2). After immersion in SN_2, the specimens were rapidly transferred to the cryochamber cold stage of a K750X Freeze Drier (Emitech LtD., UK) and freeze-dried under vacuum overnight, allowing the temperature to gradually rise from −70 °C to room temperature. The specimens were then coated with 10 nm gold by a sputter coater (108 auto/SE Cressington, UK) and imaged using the secondary electron signal by a Jeol JSM 7500FA scanning electron microscope (Jeol Ltd., Japan), equipped with a cold field emission gun and working at an acceleration voltage of 15 kV.

Peptide Reactivity with Different Aldehydes. 3,4-Dihydroxybenzaldehyde **(22)**, 2-methoxybenzaldehyde **(21)**, 3,4-dimethoxybenzaldehyde, salicylaldehyde **(2)**, piperonal **(7)**, benzaldehyde **(14)**, and isophthalic dicarboxaldehyde **(5)** were used to test the reactivity of the nematode cuticle synthetic peptide N-acetyl-EEQCTSCVQLQCP. Stock solutions of 40 mM aldehydes were prepared in 200 mM Tris-HCl buffer, pH 7.4, and that of the peptide (1 mM) in water. Fifty microliters of the reaction mixtures contained 1 mM, 5 mM, and in some cases 15 mM of the aldehyde and 0.5 mM of the peptide in 100 mM Tris-HCl buffer, pH 7.4. The reactions were performed in duplicate at 25 °C in the dark for 24 h and stopped by the addition of aqueous TFA (0.1%, final concentration). The control sample contained only the peptide at the same concentration. Ten microliters of the reaction mixtures were added with 90 μL of 0.1% TFA and immediately analyzed by HPLC-ESI-MS or stored at -20 °C until the analysis.

II. 1. f. LC-MS Detection of Reaction Products:

HPLC-ESI-MS analysis was performed using a Surveyor HPLC system (ThermoFisher, San Jose, CA, USA) connected by a T splitter to a diode array detector and to an LCQ Advantage mass spectrometer (ThermoFisher). The chromatographic column was a BioBasic-18 column (Thermo Scientific, USA), with 5 μm particle diameter (column dimensions 150 × 2.1 mm). The following solutions were utilized for the chromatographic separation: (eluent A) 0.056% aqueous TFA and (eluent B) 0.050% TFA in acetonitrile/water 80:20 (v/v). The gradient applied was linear from 0 to 40% of B in 30 min and from 40 to 100% of B in 10 min, at a flow rate of 0.30 mL/min. The T splitter addressed a flow rate of 0.20 mL/min toward the diode array detector and 0.10 mL/min toward the ESI source. During the first 5 min of separation the eluate was diverted to waste to avoid instrument damage due to the high salt concentration. The diode array detector was set at 214 and 276 nm. Mass spectra were collected every 3 ms in the positive ion mode. MS spray voltage was 5.0 kV and capillary temperature, 260 °C. Deconvolution of averaged ESI-MS spectra was automatically performed with MagTran 1.0 software.[58] Experimental mass values were compared with average theoretical values using the PeptideMass program available at http://us.expasy.org/tools. The relative abundances of the unmodified peptide and of the derivatives detected were determined by measuring the XIC (extracted ion current) peak area, when the signal/noise ratio was at least 5. The XIC analysis reveals the peak associated with the peptide of interest by searching along the total ion current chromatographic profile, the specific multiply charged ions generated at the source by the peptide. The area of the ion current peak is proportional to concentration, and under constant analytical conditions can be used for a label-free quantification.[59] For each tested aldehyde compound we calculated the relative

percentage of the XIC peak area of every derivative with respect to the sum of the XIC peak area of all the derivatives.

II. 1. g. Cysteine Reduction and Alkylation Experiments.

Twenty microliters of the reaction mixtures (with salicylaldehyde **(2)** and 3,4-dihydroxybenzaldehyde (**22)**) and of the control sample containing 10 nmol of the probe peptide was submitted to cysteine reduction at 30 °C for 1 h in 50 mM Tris-HCl buffer, pH 8.6, containing 10 Mm dithiothreitol (DTT) in a final volume of 40 µL. The reduced sample was alkylated in the dark for 45 min using 55 mM iodoacetamide (IAM), determining a Δmass value of +57.05 Da for each cysteine alkylated and subsequently analyzed by HPLC-ESI-MS.

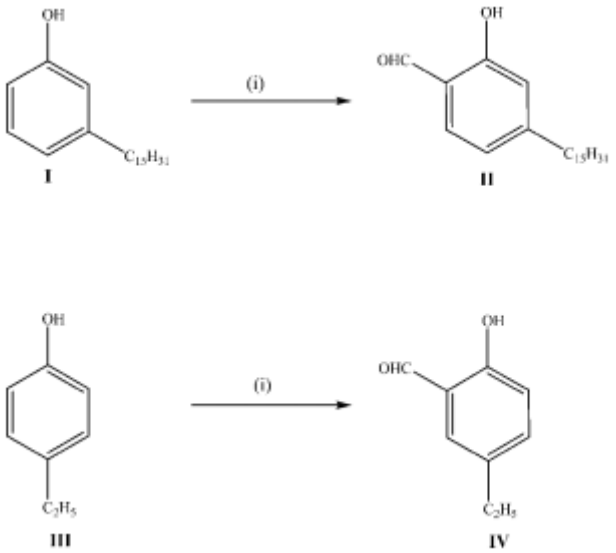


Figure 8: Reagents and conditions[a]

[a] (i) NaOH 10 N, $CHCl_3$, 65 °C, 2 h.

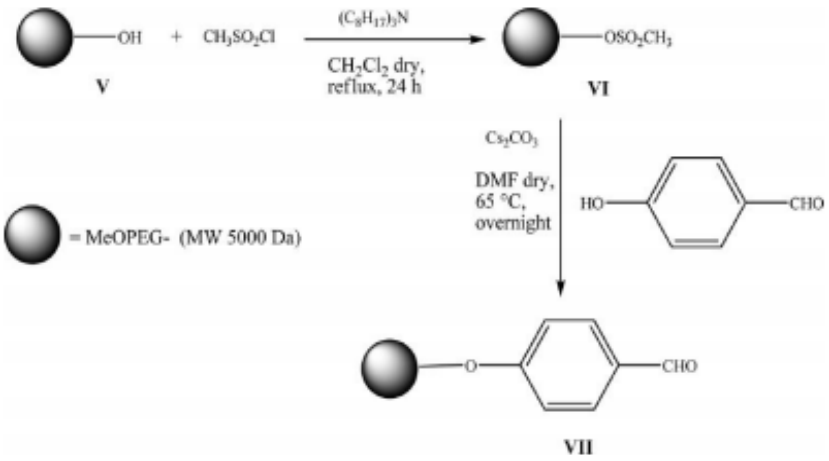


Figure 9: Reagents and conditions [a]

[a](i) mesyl chloride, trioctylamine, dichloromethane dry, reflux, 24 h. (ii) Cs_2CO_3 dimethylformamide dry, 4-hydroxybenzaldehyde, 65 °C, overnight.

II. 2. Results:

Twenty-six low molecular weight compounds **(Figure 10)** with suspected nematicidal activity were identified and purchased from commercial sources or synthetically prepared and compared for toxicity with commercial nematicide. Overall, the best performing nematicidal compounds were phthalaldehyde **(1)** and salicylaldehyde **(2)** (EC50 = 11 ± 6 and 11 ± 1 mg/L, respectively). These compounds were followed by cinnamic aldehyde and 2-naphthaldehyde (EC50 = 12 ± 5 and 13 ± 8 m g/L, respectively) and then isophthalic dicarboxyaldehyde **(5)** (EC50 = 15 ± 4 mg/L). Finally, the positive control fosthiazate, abamectin, and levamisole showed considerably higher toxicities than any experimental compounds (EC50 = 0.4 ± 0.3, 1.8 ± 0.9, and 4 ± 2 mg/L, respectively) **(Table 4)**.

The data presented here show that structural factors contribute to the widely varying toxicities of low molecular weight nematicides. With respect to the aromatic compounds, two structure-activity relationship trends are apparent. First, when no

other substituents are present in the benzene ring, the 1,2-dicarboxaldehyde was slightly more toxic if compared with isophthalic dicarboxyaldehyde. Second, the presence of a hydroxyl group in the ortho position to the formyl group conferred the highest activity if compared with the para position (4-hydroxybenzaldehyde **(11)**). In addition, when the hydroxyl group was substituted with a nitro group, the tested compound was 11 times less toxic than salicylaldehyde **(2)**.

Furthermore, when the hydroxyl group in the ortho position was glycated, as in helicin **(16)**, the test compound lost completely its activity. The same behavior was observed for the methoxylation of 2-methoxybenzoaldehyde **(21)** and vanillin **(23)**. Surprisingly, benzaldehyde **(14)** was not toxic at the tested concentration (250 mg/L), and these data are not in agreement with the previously reported findings of Ntalli,[60] probably because benzaldehyde readily undergoes oxidation by air. Moreover, the cinnamic acid **(15)** was not active if compared with the cinnamic aldehyde **(3)**. Also, the 3,4-methylenedioxyacetophenone was not active if compared with 1,3-benzodioxole-5-carbaldehyde or piperonal **(7)** (EC50=44 ± 13 mg/L). To the best of our knowledge, no information concerning the mechanism of action and the selectivity against nematodes of our aromatic aldehydes is present in the literature. One factor that most aromatic aldehydes shared is acute nematode poisoning.

The use of scanning electron microscopy (SEM) to investigate the nematode cuticle after treatment with salicylaldehyde **(2)** and (E,E)-2,4-decadienal at 100 mg/L, showed evident damages on the external cuticle, with marked macroscopic fractures **(Figure 11, compare panels A with C and F)**. The cuticles of the control nematodes showed a typical annular wrinkled pattern **(Figure 11 A, B)**, which appeared to be smooth and stretched in the nematodes treated with salicylaldehyde **(Figure 11 D, E)** and (E,E)-2,4-decadienal **(Figure 11 G, H)**, probably as a consequence of their

swollen and tumescent bodies. This diversity in the cuticle annular pattern might be caused by differences in the nematode body water content before fixation, although it is difficult to discern if the body swelling depended on a specific change in the osmolar efficiency occurring in the living nematodes and was caused by the tested compounds or by an unspecific post-mortem artifact. Intriguingly, besides these macroscopic differences, the fine morphology of the cuticle of the nematodes treated with salicylaldehyde and (E,E)-2,4-decadienal did not differ significantly from that of the control ones, not presenting microfractures **(Figure 11 B, E, H)**, and then only nanometric fracture of the nematode cuticle could be masked by the conductive gold coating.

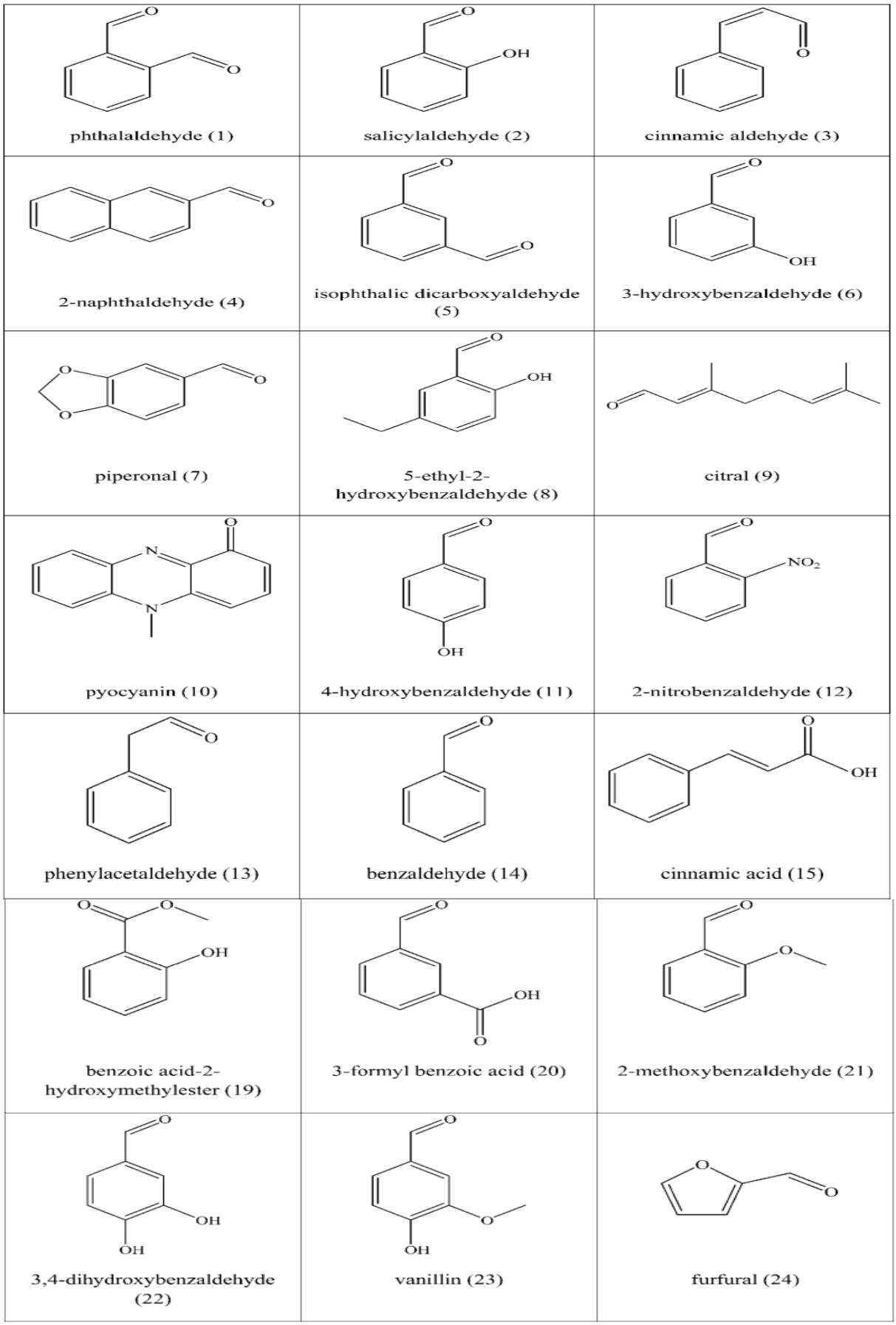


Figure 10. Chemical structures of selected nematicidal compounds.

Table 4: Values of EC100 at 1 h and EC50 at 1 Day (n=4) of Pure Compounds against *M. incognita* after Treatment

Compound	1 h	1 d
	EC_{100} (mg/L)	EC_{50} (mg/L)
phthalaldehyde (1)	>50	11 ± 6
salicylaldehyde (2)	>25	11 ± 1
cinnamic aldehyde (3)	>250	12 ± 5
2-naphthaldehyde (4)	>75	13 ± 7
isophthalic dicarboxaldehyde (5)	>100	15 ± 5
3-hydroxybenzaldehyde (6)	>250	31 ± 22
piperonal (7)	>100	44 ± 13
5-ethyl-2-hydroxybenzaldehyde (8)	>100	53 ± 22
citral (9)	>250	72 ± 27
4-hydroxybenzaldehyde (11)	500	75 ± 23
2-nitrobenzaldehyde (12)	>200	120 ± 25
phenylacetaldehyde (13)	1000	167 ± 45
benzaldehyde (14)	>100	>250
cinnamic acid (15)	>250	>250
helicin (16)	>250	>250
3,4-methylendioxy acetophenone (17)	>500	>500
catechol (18)	>500	>500
2-hydroxy-4-pentadecylbenzaldehyde (II)	>1000	>1000
benzoic acid-2-hydroxy methyl ester (19)	>1000	>1000
PEG-O-4-hydroxybenzaldehyde (VII)	>1000	>1000
3-formylbenzoic acid	>1000	>1000

2-methoxybenzaldehyde (21)	>1000	>1000
3,4-dihydroxybenzaldehyde (22)	>1000	>1000
3,4-dimethoxybenzaldehyde	>1000	>1000
vanillin (23)	>1000	>1000
ouabain	>1000	>1000
pyocyanin (10)	>250	72 ± 25
fosthiazate	3 ± 1	0.4 ± 0.3
abamectin	>75	2 ± 1
levamisole	>20	4 ± 2
(E,E)-2,4-decadienal	>20	12 ± 2
furfural[a] (24)	11 ± 1	8 ± 1
oligomycin	>500	>500

[a] This compound showed a strong fumigant activity

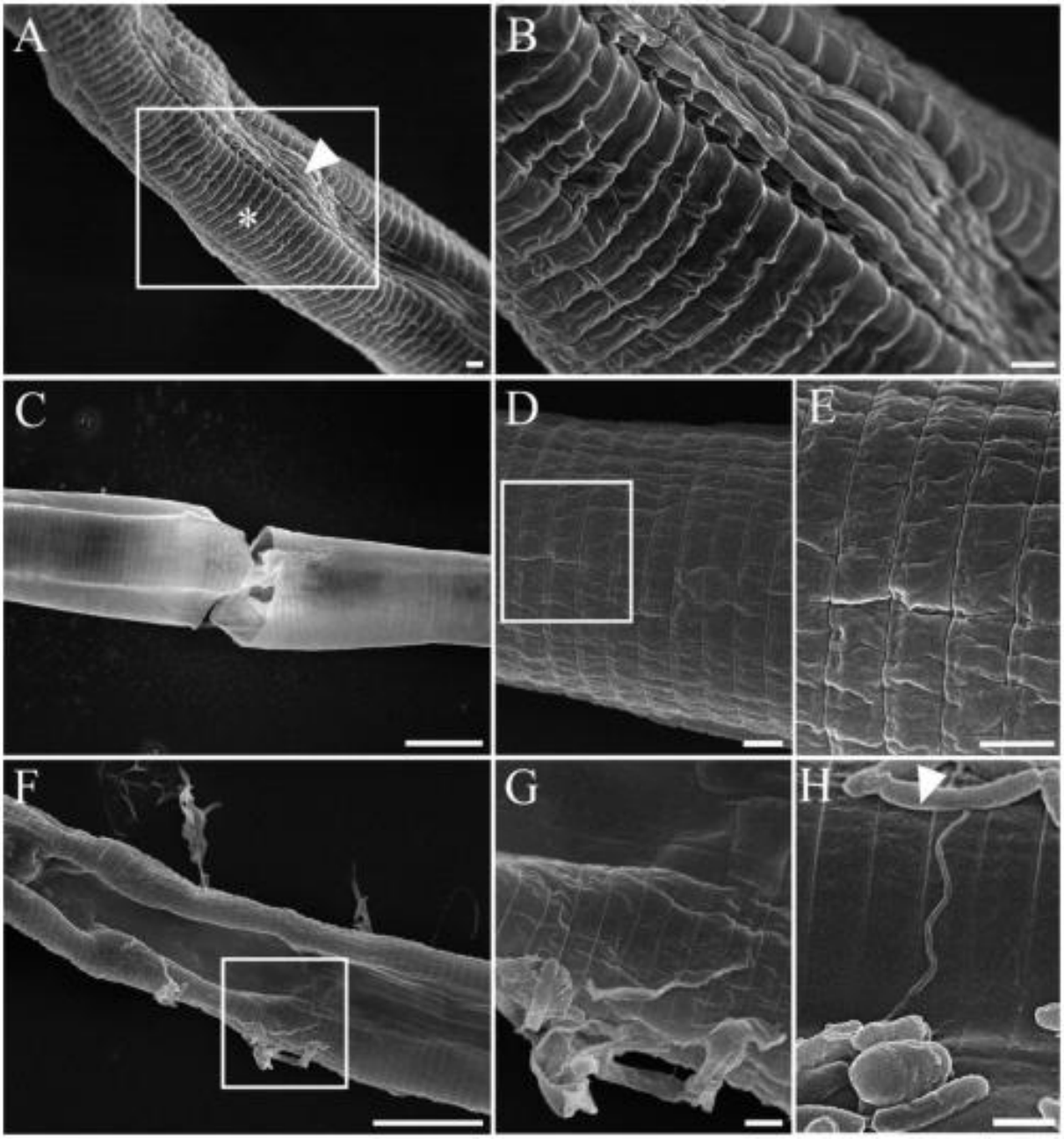


Figure 11: Scanning electron microscopy (SEM) micrographs of the cuticle of *Meloidogyne incognita* juveniles. (A, B) Untreated (control) specimens: (A) low magnification of a portion of *M. incognita* juvenile body surface (note the annulated cuticle cortical layer (asterisk) separated by several epicuticular longitudinal ridges (arrowhead)); (B) detail of the cuticle correspondent to the region boxed in panel A. (C−E) Body surface after treatment with salicylaldehyde (2): (C) low magnification showing a macroscopic fracture through the nematode body wall; (D) detail of the cuticle annular pattern; (E) higher magnification of several adjacent annuli in the cuticle region boxed in panel D. (F−H) Body surface after treatment with (E,E)-2,4-decadienal; (F) low magnification of the nematode body surface showing conspicuous damages at the cuticular layers; (G) detail of the damaged cuticle annular region boxed in panel F; (H) higher magnification of the cuticle annular region (note several rod-shaped bacteria on adjacent cuticular annuli (arrowhead)). Scale bars are 1 μm for panels A, B, D, E, G, and H and 10 μm for panels C and F.

Furthermore, considering that 4-hydroxybenzaldehyde **(11)** was active against juveniles of *M. incognita* but the nature of this toxicity is mostly undefined, we prepared a structurally related compound with the same aromatic scaffold bearing a bulky PEGylated group in the para position. In *in vitro* tests, this compound did not show any activity, thus suggesting it was not able to diffuse through the nematode cuticle, most likely signifying an internal biochemical target of tested aldehydes.

Taking into account the nematode toxicity experiments with different aldehydes and the macroscopic cuticle fractures observed by SEM, we hypothesize that the potential mechanism of action of the tested aromatic aldehydes might be related to the alteration of the external cuticle or an internal biochemical target might be involved. First, to understand the toxicity mechanism, we decided to prove the formation of a covalent adduct between aromatic aldehydes and endogenous proteins and/or peptides mimicking the nematode cuticle.

The nematode cuticle, well reviewed in the *Caenorhabditis elegans* model,[61] is a multifunctional exoskeleton. The cuticle collagens are encoded by a gene family with over 170 members.[62] They have a characteristic tripeptide structure of short interrupted blocks of glycine-proline-hydroxyproline sequence flanked by conserved cysteine residues and can be grouped into families according to homology.[63] Taking into account the feasible reaction of small molecules having electrophilic properties with nucleophilic amino acid residues of the proteins, that is, cysteine, we investigated the chemical reaction between some selected compounds and the nematode's cuticle peptides to better understand their potential toxicity.

Reactivity of electrophilic aldehydes toward cuticle or skin peptides has been investigated qualitatively for individual chemicals by directly detecting and

characterizing adducts between the peptides and the chemical using liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR).[64,65]

With an LC-MS platform, constituents of a reaction mixture can be separated, and full scan mass spectra for ions with mass/charge ratio (*m/z*) are acquired during the chromatographic run. Consequently, an LC-MS analysis of the products after incubation with peptide and test compounds allows selective monitoring of the test compound and testing of different molecular weight adducts. These approaches are widely used to predict skin sensitization of test compounds toward skin peptides and proteins.[66] In parallel, it was assayed by cysteine reduction/alkylation experiments, the involvement of the cysteine residues in the adduct formation between the tested compounds and the peptide.

The sequence of the probe peptide utilized in the assay corresponded to the 100-112 residues of collagen 3 (Swiss-Prot code O01945) present in the cuticle of *M. javanica* and coded by the Mjcol-3 gene.[67] The peptide sequence was chosen according to the following criteria: (1) it covered a region of Mj collagen 3 containing from three to five cysteine residues involved in the disulfide bridge formation during the collagen assembly;[67] (2) cysteine or lysine containing peptides are usually used for mechanistic understanding of the reactivity of chemicals toward collagen peptides in skin sensitization experiments.[68]

At the concentration of 1 mM adduct formation was observed for the following compounds; 3,4-dihydroxybenzaldehyde **(22)**, 2-methoxybenzaldehyde **(21)**, 3,4-dimethoxybenzaldehyde, and salicylaldehyde **(2)**. Benzaldehyde **(14)**, isophthalic dicarboxyaldehyde **(5)**, and piperonal **(7)** gave a total adduct percent of <5 **(Table 5)**.

The observation that 2-methoxybenzaldehyde **(21**) and 3,4-dimethoxybenzaldehyde were not active against *M. incognita* in *in vitro* experiments led us to hypothesize a different and unspecific mechanism of action rather than an effect on the external cuticle of nematodes.

Table 5. Reactivity of the Peptide with Different Aldehydes: Relative Percentage of Total Adducts ((Monomeric + Dimeric) × 100/ (Monomeric + Dimeric + Free Peptide)) [a]

Compd	1 mM	5 mM	15 mM
2-methoxybenzaldehyde	74	77	NT
3,4-dihydroxybenzaldehyde	72	89	73
3,4-dimethoxybenzaldehyde	65	70	NT
salicylaldehyde	61	57	NT
piperonal	5	30	NT
isophthalic dicarboxaldehyde	1	2	0
benzaldehyde	0	0	6

Peptide experimental average mass value, determined by HPLC-ESI-MS analysis, was 1508.7± 0.3 Da, in agreement with the theoretical mass value of 1508.6 Da expected for the reduced form. Furthermore, HPLC-ESI-MS analysis revealed the presence of oxidized derivatives of the peptide generated by intra or intermolecular disulfide bridges. The reaction products of the peptide with different aldehydes, analyzed by HPLC-ESI-MS, are reported in **Table 6**. The mass values were in agreement with (a) the formation of monomeric and dimeric adducts with the aldehydes; (b) the binding of one, two, or three aldehyde moieties to the peptide depending on the reactivity of the aldehyde; and (c) the presence of acetal linkages in the adducts (observed Δ mass = mass value of aldheyde -18 Da). Such adduct

formation can be linked to the reactivity between the peptide and test chemical, whereas peptide depletion often indicates peptide dimerization or other oxidative processes.

As far as it concerns the different reactivities to the aldehydes, the peptide bound up to two 3,4-dihydroxybenzaldehyde **(22)** and 2-methoxybenzaldehyde **(21)** moieties in the monomeric adducts and up to three moieties in the dimeric adducts, but the peptide bound up to two salicylaldehyde **(2),** 3,4-dimethoxybenzaldehyde, and piperonal **(7)** moieties in both monomeric and dimeric derivatives and only one benzaldehyde moiety in both the monomeric and dimeric peptide derivatives. In the reaction with isophthalic dicarboxaldehyde the peptide generated only the monomeric 1:1 adduct.

Cysteine reduction/alkylation experiments showed that the reaction involved the cysteine residues. Indeed, in the monomeric adduct linking one salicylaldehyde **(2)** (or 3,4-dihydroxybenzaldehyde **(22)**) moiety, only two cysteines were alkylated, and in that one linking two 3,4-dihydroxybenzaldehyde **(22)** moieties only one cysteine was alkylated. The monomeric adduct was never observed with all three cysteine residues alkylated, confirming the involvement of the cysteine residues in the formation of thioacetal linkages with the aldehydes. Following the cysteine alkylation, the dimeric adducts linking one, two, or three aldehyde moieties showed four, two, or no alkylated cysteines, respectively. This finding suggests that the dimeric adducts are dithioacetals generated by the cross-linking of the aldehyde with two cysteine residues belonging to different peptide chains.

Moreover, quantitative analysis evidenced that the major peptide reactivity was toward 3,4-dihydroxybenzaldehyde **(22)**, 3,4-dimethoxybenzaldehyde, and

2-methoxybenzaldehyde **(21)**, followed by salicylaldehyde **(2)**, piperonal **(7)**, benzaldehyde **(14)**, and isophthalic dicarboxaldehyde **(5)**; these last generated the lowest quantity of adducts also at the higher concentration tested (15 mM). A plausible explanation might be related to the fact that the reaction between the sulfur large-sized nucleophile and the carbonyl carbon atom can be considered as a soft-soft interaction, mainly orbital controlled. Thus, conversely to the acetalization process, which is charge-controlled, thioacetalization reaction is energetically favored when electron-rich carbonyl substrates are employed.[69] Besides, in relation to the high molecular orbital coefficient at the carbonyl carbon atom, the large orbital of the sulfur nucleophile may overlap efficiently, so satisfying the Bürgi−Dunitztrajectory.[70]

A mechanism for monomer adduct formation is depicted in **Figure 12**. A possible proton transfer to the oxyanionic form of the thiohemiacetal from the amide backbone and the subsequent formation of a (S,N) cyclic acetal is proposed.[71] The relative abundances of total adducts generated are reported in **Table 6**. The major products of the reaction were the dimeric adducts. This might probably be due to the fact that the sulfur of the −SH residue is a better electron donor to an intermediate thiocarbenium ion than the nitrogen. The higher percentages of dimeric adducts were observed in presence of 5 mM 3,4-dihydroxybenzaldehyde **(22)** (76.0%), followed by 3,4-dimethoxybenzaldehyde (65%), 2-methoxybenzaldehyde **(21)** (57%), salicylaldehyde **(2)** (54%), and piperonal **(7)** (27%).

Unfortunately, it was not observed for the tested compounds a correlation between reactivity and potency because other factors, such us cuticle penetration or the activity against other biochemical targets, may be involved.

It is well-known that glycation of biological proteins can lead to the formation of advanced glycation end products (AGEs) that have a propensity to generate toxic reactive oxygen species (ROS).[72] In the same fashion, gossypol, a triterpenoid aldehyde, isolated from cotton plants was shown to inhibit mitochondrial electron transfer and stimulate generation of ROS.[73] Thus, for these reasons it was hypothesized that ROS generation produced by redox-active aldehydes may have a crucial role in the inhibition of vacuolar-type proton translocating ATPase (V-ATPase). The latter target is an enzyme complex that pumps protons across membranes, energized by ATP hydrolysis. V-ATPases, depending on their subcellular localization and orientation in the membrane, can acidify intracellular compartments[74] or the extracellular environment.[75] Consequently they are involved in nematode nutrition osmoregulation, cuticle synthesis, neurobiology and reproduction.[76]

Osmoregulation can be critical for nematodes as maintenance of homeostasis and purging of toxins would be essential normal and hostile environments.[77] On the basis of in vitro studies, V-ATPases from mammals, fungi, and plants are subject to oxidative inactivation, where as activity can be restored by using a reducing agent.[78] Moreover, oxidative inhibition of the V-ATPase is ascribed to disulfide bond formation between conserved cysteine residues at the catalytic site.[79] Furthermore, V-ATPase is inhibited by the antibiotics bafilomycin A1 and concanamycin A and inactivated by the phenazine pyocyanin **(10)**.[80] In fact, when this latter was tested against *M. incognita* J2, an EC50 at 24 h of 72 ± 25 mg/L was found. This toxicity against J2 was comparable with structurally similar compounds such as 2-pyridinecarboxaldehyde, indole-3-carboxyaldehyde, and pyrrole-2-carboxaldehyde (3.60 ± 1.16, 301 ± 33 and 392 ± 32 mg/L, respectively).

Consistent with previous works[81] we observed that J2 nematodes treated with pyocyanin and tested aldehydes were paralyzed or died filled with liquid in a straight shape. Liegeois observed that *C. elegans* lacking larval expression of the V-ATPase genes often fill with fluid and die,[82] which mimics the phenotype observed by Nelsons when they ablated nematode excretory pore.[83] All of these facts suggest that the tested aromatic aldehydes probably act by inhibiting the nematode V-ATPase enzyme.

Moreover, Mahajan-Miklos et al. reported that pyocyanin was responsible for the fast killing of the nematode *C. elegans* by producing reactive oxygen intermediates, and this effect was exacerbated by high levels of osmolarity.[84] It is noteworthy that when ouabain, an inhibitor of the plasma membrane Na^+/K^+ ATPase, was tested against *M. incognita* J2, we did not observe any activity. Additionally, Kim et al. reported that *o*-hydroxybenzaldehydes such as 2-hydroxy-5-methoxybenzaldehyde and *o*-vanillin showed strong anti-fungal activity against filamentous fungi.[85]

Moreover, these authors showed that when oxidative stress agents, such as *o*-hydroxybenzaldehyde, were applied to sakA Δ and mpkCΔ MAPK mutant strains of *A. fumigatus*, they became susceptible because the mutated MAPK system was incapable of launching a fully operational oxidative stress response.[85]

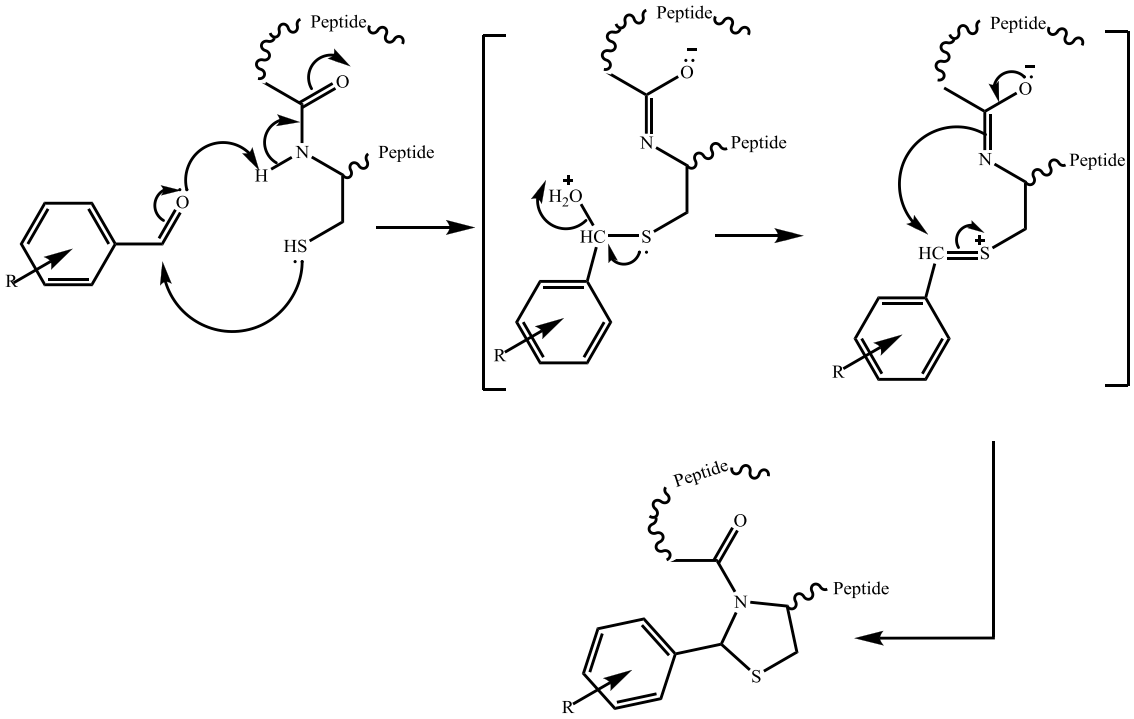


Figure 12: Possible Reaction Mechanism for Monomer Adduct Formation

Table 6: Adducts Generated by the Reaction of MjCol3 Peptide with Various Aldehydes at Different Concentrations: Experimental and Theoretical Average Mass (Mav) Values (Da) of the Products of Reaction, Ratio of Peptide/Linked Aldehydes, and Its Relative Percentage[a]

Aldehyde	**Reaction products Exp. Mav. (Th. Mav)**	**Attribution**	**Peptide/aldehyde**	**1 mM**	**5 mM relative %**	**15 mM**
2-methoxybenzaldehyde	1624.7 ± 0.3 (1626.8)	Monomeric adduct*	(1:1)	**74** = 12	77 = 18	
	1744.3 ± 0.3 (1744.9)	Monomeric adduct	(1:2)	0	2	
	3132.6 ± 0.6 (3135.4)	Dimeric adduct*	(2:1)	42	27	NT
	3253.1 ± 0.6 (3253.5)	Dimeric adduct	(2:2)	20	21	
	3371.4 ± 0.6 (3371.7)	Dimeric addcut	(2:3)	0	9	
3,4-dihydroxybenzaldehyde	1626.7 ± 0.3 (1628.7)	Monomerci adduct*	(1:1)	**72** = 5	**89** = 13	**73** = 12
	1749.2 ± 0.3 (1748.8)	Monomeric adduct	(1:2)	0	0	2
	3135.4 ± 0.6 (3137.3)	Dimeric addcut*	(2:1)	40	37	25
	3256.8 ± 0.6 (3257.4)	Dimeric adduct*	(2:2)	27	35	31
	3378.4 ± 0.6 (3377.6)	Dimeric adduct	(2:3)	0	4	3

	1654.9 ± 0.3 (1656.8)	Monomeric addcut*	(1:1)	**65** = 7	**70** = 6	
3,4-dimethoxybenzaldehyde	3163.4 ± 0.6 (3165.4)	Dimeric adduct*	(2:1)	44	42	NT
	3313.0 ± 0.6 (3313.6)	Dimeric adduct	(2:2)	14	22	
	1610.7 ± 0.3 (1612.7)	Monomeric adduct*	(1:1)	**61** = 9	**57** = 3	
salicylaldehyde	3118.7 ± 0.6 (3121.3)	Dimeric adduct*	(2:1)	52	42	NT
	3222.9 ± 0.6 (3225.4)	Dimeric adduct*	(2:2)	0	12	
	1640.6 ± 0.3 (1640.7)	Monomeric adduct	(1:1)	**5** = 1	**30** = 4	
piperonal						NT
	3147.3 ± 0.6 (3149.3)	Dimeric adduct*	(2:1)	4	26	
isophthalic dicarboxaldehyde	1624.1 ± 0.3 (1624.7)	Monomeric adduct	(1:1)	0.7	1.6	0
	1596.5 ± 0.3 (1596.7)	Monomeric adduct	(1:1)			**6** = 1
benzaldehyde				0	0	
	3105.6 ± 0.6 (3105.3)	Dimeric adduct	(2:1)			5

III. Nematicidal Activity of 2-Thiophenecarboxaldehyde and Methylisothiocyanate from Caper (*Capparis spinosa*) against *Meloidogyne incognita*:

III. 1. *Capparis spinosa*:

Caper *(Capparis spinosa)* is a perennial winter-deciduous plant native to the Mediterranean region and is also widely grown in the dry regions in west and central Asia. Its immature flower buds, unripe fruits, and shoots are consumed as foods or condiments in cooking.[86] The shrubby plant is many-branched, with alternate leaves, thick and shiny, round to ovate in shape. The flowers are complete, sweetly fragrant, showy, with four sepals, and four white to pinkish-white petals, many long violet-colored stamens, and a single stigma usually rising well above the stamens **(Figure 13)**. Different parts of this plant, including the flower buds, fruits, seeds, shoots, and bark of roots, were traditionally used as culinary spices and folk medicines like anti-inflammatory agents, to treat earache and cough, and to expel intestinal worms.[87] Both buds and the fruit (caper berry) are usually consumed pickled. Studies on the chemical composition of *C. spinosa* have reported the presence of alkaloids, lipids, polyphenols, flavonoids, and glucosinolates.[88] Furthermore, *C. spinosa* extract was reported to be rich in flavonoids such as kaempferol, rutin, quercetin, and quercetin derivatives[89], which are known to have antiallergic, anti-inflammatory, and antioxidant proprieties[90]. This plant is also rich on aliphatic glucosinolates.[91] Aliphatic glucosinolates, belonging to sulfur secondary metabolites group, are hydrolyzed by the vacuolar enzyme myrosinase (thioglucoside glucohydrolase, EC 3. 2. 3. 1) to produce isothiocyanates, nitriles, and thiocyanates in addition to glucose and sulfate ion. Isothiocyanates are general biocides whose activity is based on irreversible interactions with proteins.[92]

Figure 13: Caper plant

III. 2. Materials and Methods:

III. 2. a. Chemicals:

Methylisothiocyanate, 2-naphtaldehyde, 2-thiophenecarboxaldehyde, 2,5-thiophenedicarboxyaldehyde, 2-pyridine carboxaldehyde, indole-3-carbonitrile, indole-3-carboxaldehyde, 1H-indole-2,3-dione, pyrrole-2-carboxaldehyde, 2-pyrrolidone, N-methyl-2-pyrrolecarboxaldehyde, methanol, Tween, and DMSO were purchased from Sigma Aldrich (Milan, Italy) with a purity >98-99%. Water was distilled through a Milli-Q apparatus.

III. 2. b. Experimental Chemistry:

Reaction progress was monitored by TLC using Aldrich silica gel 60 F254 (0.25 mm) plates. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Unity Inova 500 MHz

spectrometer. IR spectra were recorded in Nujol mull or neat on a Perkin-Elmer 1310 spectrophotometer. Melting points were determined on a Stuart Scientific SMP 11 melting point apparatus.

III. 2. c. Synthesis of 2,5-Thiophenedicarbonitrile:

A mixture of 2,5-thiophenedicarboxaldehyde (2.14 mmol, 0.30 g) and NH_2 OH·HCl (6.42 mmol, 0.45 g) in pyridine (22.26 mmol, 1.8 mL) was stirred at 100 °C for 2.5 h. After reaction, the solvent was removed under vacuum to give the crude products as a brown viscous liquid. Yield (%) = 55. IR (Nujol mull): 3280, 2320 cm^{-1}. ^{1}H NMR (500 MHz, DMSO): δ 7.65 (s, 2H) ppm. ^{13}C NMR (500 MHz, DMSO): δ 145.11, 119.67, 110.34 ppm.

III. 2. d. Synthesis of 5-Formylthiophene-2-carbonitrile:

A mixture of 2,5-thiophenedicarboxaldehyde (2.14 mmol, 0.30 g) and NH_2OH·HCl (3.21 mmol, 0.22 g) in pyridine (18.58 mmol, 1.5 mL) was stirred at 100 °C for 2.5 h. After reaction, solvent was removed under vacuum to give the crude products as an orange solid, which was recrystallized from ethanol. Yield (%) = 96. Mp = 198−200 ° C. IR (Nujol mull):

3300, 2780, 2365, 1675 cm^{-1}. ^{1}H NMR (500 MHz, DMSO): δ 12.08 (s, 1H), 7.87 (d, 1H, J = 4.0 Hz), 7.43 (d, 1H,J = 4.0 Hz) ppm. ^{13}C NMR (500 MHz, DMSO): δ 182.11, 154.78, 139.54, 134.89, 120.00, 111.18 ppm.

III. 2. e. Synthesis of 2-Thiophenecarboxaldehyde oxime:

A mixture of 2-thiophenecarboxaldehyde (8.93 mmol, 1 g) and hydroxylamine chlorhydrate (13.4 mmol, 0.93 g) was stirred at 100 °C for 1 h. After reaction, 15 mL of distilled water was added and the reaction mixture was extracted with ethyl acetate

(3× 15 mL). The organic phase was dried over sodium sulfate, and the solvent was removed under vacuum to give the product as a viscous pale green oil. Yield (%) = 62. IR (Nujol mull): 3270, 2570, 1635 cm^{-1}. ^{1}H NMR (500 MHz, DMSO): δ 9.04 (bs, 1H), 7.54 (d, 1H, J = 4.5 Hz), 7.22 (d, 1H,J = 5.3 Hz), 7.12 (m, 1H) ppm. ^{13}C NMR (500 MHz, DMSO): δ 153.12, 144.12, 132.76, 122.67, 117.80 ppm.

III. 2. f. Synthesis of Thiophene-2-carbonitrile (F):

A mixture of 2-thiophenecarboxaldehyde oxime (1.97 mmol, 0.25 g), methanesulfonyl chloride (1.97 mmol, 0.155 mL), and dry acid alumina was heated in an oil bath at 100 °C for 1 h. After reaction, 15 mL of ethyl acetate was added and the resulting mixture was filtered to remove alumina. The filtrate was washed with distilled water (2 × 10 mL), and the organic phase, previously dried on sodium sulfate, was concentrated to give the crude product as an oil. The pure product, as a yellow oil, was obtained after flash chromatography on silica gel (eluent dichloromethane).

Yield (%) = 61. IR (neat): 3250, 2780, 2374 cm^{-1}. ^{1}H NMR (500 MHz, DMSO): δ 12.08 (s, 1H), 7.87 (d, 1H, J = 4.0 Hz), 7.43 (d, 1H,J = 4.0 Hz) ppm. ^{13}C NMR (500 MHz, DMSO): δ 182.11, 154.78, 139.54, 134.89, 120.00, 111.18 ppm.

III. 2. g. Synthesis of 2-Pyridinecarboxaldehyde Oxime:

A mixture of 2-pyridinecarboxaldehyde (9. 34 mmol, 1 g) and hydroxylamine chlorhydrate (14 mmol, 0.97 g) was stirred at 100 °C for 1 h. After reaction, 15 mL of distilled water was added; the reaction mixture was neutralized and then extracted with ethyl acetate (3 × 15 mL). The organic phase was dried over sodium sulfate, and the solvent was removed under vacuum to give the product as a viscous pale yellow

solid. Yield (%) = 70. Mp = 110-112 °C. IR (Nujol mull): 3280, 2550, 1645 cm^{-1}. ^{1}H NMR (500 MHz, CDCl3): δ 9.47 (bs, 1H), 8.64 (d, 1H, J = 4.5 Hz), 8.32 (s, 1H), 7.83 (d, 1H, J = 7.5 Hz), 7.72 (m, 1H), 7.29 (m, 1H) ppm. ^{13}C NMR (500 MHz, $CDCl_3$): δ 152.51, 149.04, 144.20, 136.12, 127.00, 123.73 ppm.

III. 2. h. Synthesis of Pyridine-2-carbonitrile:

A mixture of 2-pyridinecarbaldehyde oxime (2.46 mmol, 0.30 g), methanesulfonyl chloride (2.46 mmol, 0.19 mL), and dry acid alumina was heated in an oil bath at 100 °C for 1 h. After reaction, 15 mL of ethyl acetate/acetonitrile (1/1) was added and the resulting mixture was filtered to remove alumina. The filtrate was washed with distilled water (2 × 10 mL), and the organic phase, previously dried on sodium sulfate, was concentrated to give the crude product as an oil. The pure product, as a yellow oil, was obtained after flash chromatography on silica gel (eluent hexane/ethyl acetate 1/1). Yield (%) = 67. IR (neat): 3690, 2235 cm^{-1}. ^{1}H NMR (500 MHz, CDCl3): δ 7.75 (m, 2H), 7.22 (d, 1H, J = 4.5 Hz) ppm. ^{13}C NMR (500 MHz, $CDCl_3$): δ 137.32, 132.60, 127.45, 114.21, 109.91 ppm.

III. 2. i. Plant Materials:

The aerial parts and buds of *C. spinosa* were collected in Cagliari, Sardinia, in February 2012. The extraction was made as previously mentioned then was analyzed and by GC-MS. Plant materials were dried at 105 °C for 24 h. The moisture of the stems, leaves, and buds were 51.18%, 79.12%, and 79.60% respectively.

III. 2. j. GC-MS Analysis:

Chromatographic separation and identification of the main components of methanol extracts of *Capparis spinosa* stems (SME), leaves (LME) and buds (BME) was

performed on a Trace GC Ultra Gas Chromatograph (Thermo Finni gan, MA) coupled with a Trace DSQ mass spectrometry detector, a split-splitless injector, and an Xcalibur MS platform. The column was a CP-WAX 57CB from Varian (60 m long, 0.25 mm i.d., and 0.25 μm film thickness; Varian Inc., USA). The injector and transfer line were at 200 °C. The oven temperature was programmed as follows: 50 °C (hold 1 min) then raised to 220 °C (3 °C/min) and isothermally hold for 13 min. Helium was the carrier gas at a constant flow rate of 1 mL/min; 1 μL of each sample was injected in the splitless mode (60 s). Mass spectrometry acquisition was carried out using the continuous (EI positive) scanning mode from 45 to 500 amu. *C. spinosa* methanol extract components were identified by (a) comparison of their relative retention times and mass fragmentation with those of authentic standards and (b) computer matching against a NIST/EPA/NIH Mass Spectral Library (NIST 08). For stems, leaves, and buds quantitative analysis of methylisothiocyanate and 2-thiophenecarboxaldehyde were carried out in triplicate with the external standard method. Extracts and compounds found are used to test their nematicidal activity against *M. incognita* J2.

III. 3. Results:

As a result of the GC-MS analysis, we were able to identify 24 plant metabolites of the methanol extract of stem **(Table 7)**. We confirmed by co-chromatography the presence of 2-thiophenecarboxaldehyde in the SME, and to our knowledge, this is the first report of this compound found in a plant extract. We found 2-thiophenecarboxaldehyde in stem extract at a concentration of 1.12 ± 0.31 mg/kg, while methylisothiocyanate levels in stem, leaves, and buds were 116 ± 42, 7.33 ± 3.78, and 3.87 ± 0.98 mg/kg on a dry weight basis, respectively. SME was active against *M. incognita* at an $EC_{50/3d}$ of 215 mg/L **(Table 8)**. This value is rather low

considering the activities of *R. chalepensis* methanol extracts against *M. incognita*, exhibiting an EC_{50} value of 1001 mg/L after 1 day of J2 immersion in test solutions.[52] Under the same conditions extracts from leaves and buds had no effect at all the tested concentrations (10 −1000 mg/L).

Among secondary plant metabolites identified in the methanol extract of different parts of the plant *C. spinosa*, methylisothiocyanate, furfural, and 2-thiophenecarboxaldehyde were the most active compounds against J2 with $EC_{50/1d}$ of 7.86, 8.50, and 14.08 mg/L, respectively. Moreover, 2-pyridinecarboxaldehyde and 2-thiophenecarboxaldehyde showed a fumigant activity with an $EC_{50/1d}$ of 62 and 100 mg/L respectively. The activity of methylisothiocyanate and 2-thiophenecarboxaldehyde against *M. incognita* is rather high if compared to the nematicidal activity of fosthiazate ($EC_{50/1d}$ = 0.40 mg/L) **(Table 9)**. This is the first report of the irreversible nematicidal activity of 2-thiophenecarboxaldehyde as a constituent of *C. spinosa* against *M. incognita*. According to our results, methylisothiocyanate was the principal nematicidal constituent of *C. spinosa* for all extracts (SME, LME, BME) with percent concentrations of 64.57%, 17.56%, and 2.13%, while 2-thiophenecarboxaldehyde was found only in the stem extract (SME) at a 0.19% concentration. This evidence supports the fact that leaves and buds extracts are not active in *in vitro* experiments against J2. Methylisothiocyanate is also responsible for the biological activity of *Boscia senegalensis* (Pers) Lam. ex Poiret (*Capparaceae*) fruits and leaf extracts against stored grain insects.[93] Serra et al. reported that 2-phenyethylglucosinolate and its hydrolysis derivatives from water cress (*Nasturtium officinale* L.) were active against the potato cyst nematode *Globodera rostochiensis* (Woll.)[94] which support the fact of nematicidal activity of isothiocyanates. Considering that aldehydes such as furfural and

2-thiophencarboxaldehyde were active against juveniles of *M. incognita*, we prepared structurally related compounds with the same aromatic scaffold but supporting selected functional groups such as oxime and nitriles **(Figure 14)**. The *in vitro* experiments against J2 evidenced the interesting biological activity of 2,5-thiophenedicarboxaldehyde, 2-pyridincarboxaldehyde, 2-thiophenecarboxaldehyde, and indole-3-carbonitrile with EC_{50} of 1.13, 3.60, 14.08 and 68.9 mg/L, respectively **(Table 9)**. Together with methylisothiocyanate and furfural, 2-thiophenecarbaxoaldehyde and 2,5-thiophenecarbaxoaldehyde were the most active compounds tested against J2 while their derivatives 5-formylthiophenecarbonitrile and 2-thiophenecarboaxaldehyde oxime were not active at the tested concentration. Interestingly, indole-3-carbonitrile was 4-fold more potent than indole-3-carboxaldehyde. Pyrrole-2-carboxaldehyde showed an $EC_{50/1d}$ of 392 mg/L, while its N-methylderivative N-methyl-2-pyrrolecarboxaldehyde was inactive.

Table 7: GC-MS Analysis of the Methanolic Extract of Caper Stems (SME)

compound	R_t	relative %	mw	EIMS (m/z) (amu) (abundance)
methylisothiocyanate	16.17	64.57	73	73 (100); 72 (52); 70 (12); 45 (7); 58 (7)
methyl pyrazine	18.09	0.84	94	94 (100); 67 (45); 53 (15)
3-hydroxy 2-butanone	19.24	0.43	88	45 (100); 88 (30); 73 (10); 55 (5)
unknown	19.83	1.48		74 (100); 45 (18); 74 (10)
2,5-dimethyl pyrazine	20.47	0.05	108	108 (100); 72 (38)
2,3-dimethyl pyrazine	21.22	0.15	108	108 (100); 67 (90)
4-hydroxy-4-methyl-2-pentanone	22.04	1.88	116	59 (100); 101 (55)
o-methyl-*n*-methyl carbamate	24.90	3.93	89	58(100); 89(55); 74(42); 59(22)
furfural	25.92	3.62	96	96 (100); 95 (59); 67 (10)
2-furyl methyl ketone	27.60	0.16	110	95 (100); 110 (33)
5-methyl-2-furaldehyde	30.37	0.67	110	110 (100); 108 (88); 53 (45); 81 (10)
benzoic acid methyl ester	32.23	0.28	136	105 (100); 77 (48); 136 (47); 51 (15)
furfuryl alcohol	33.64	1.00	98	98 (100); 97 (52); 69 (30); 81 (28); 70 (24)
2-(1*H*-imidazol-5-yl)propan-1-amine	34.39	0.06	125	96 (100); 95 (83); 68 (30); 59 (22); 81 (10)
3-thiophenecarboxaldehyde	34.53	0.22	112	111 (100); 112 (95); 83 (20); 58 (10); 57 (9)
2-thiophenecarboxaldehyde	35.10	0.19	112	111 (100); 112 (75); 58 (11); 57 (8); 83 (7)
(5-methyl-2-furyl)methanol	35.94	0.18	112	95 (100); 112 (72); 97 (40); 69 (32); 53 (20)
unknown	36.69	0.07		138(100); 123(90); 95(35);63 (18)
unknown	37.40	4.13		59 (100); 60 (3); 93 (2)
β-damascenone	39.67	0.12	190	69 (100); 121 (60); 190 (30); 105 (28); 91(15); 77(12); 79(8)
unknown	42.30	3.45		105 (100); 74 (20); 58 (15); 60 (13); 72 (11); 75 (9); 61 (7)
unknown	43.60	0.53		71(100); 55 (68)
methyl 2-pyrrolyl ketone	44.98	0.32	109	94(100); 109(82); 66(55)
unknown	45.48	0.23		127(100); 55(13); 99 (10)
formic acid. allyl ester	51.62	1.16	86	57(100); 58(43)
4-acetoxy-3-methoxystyrene	52.27	0.22	192	150(100); 135(63); 107(30); 77(22)
2.3-dihydro-3.5-diidroxy-6-methyl 4*H*-piran-4-one	54.49	0.59	144	144(100); 101(65); 72(32); 55(28); 73(20)
thioacetamide	56.99	0.32	75	75(100); 59(68)
5-hydroxymethyl-2-furan carboxaldehyde	61.54	8.08	126	97(100); 126(52); 69(32)
2-thiophene ethanol	64.20	1.06	128	97(100); 128(50); 69(32)

Table 8: Nematicidal Activity of *C. spinosa* Extracts (SME, LME, and BME) against *M. incognita*

Extract	$EC_{50/1d}$ (mg/L ± SD)	$EC_{50/3d}$ (mg/L ± SD)
SME	>1000	215 ± 36
LME	>1000	>1000
BME	>1000	>1000

[a] *C. spinosa* methanolic extracts of stem, SME; leaves, LME; and buds, BME.

Table 9: EC_{50} Values (mg/L ± SD, n = 4) of Pure Compounds Against *M. incognita* at 1 h and 1 Day After Treatment

Compound	**1 h**	**1 d**
	EC_{50} (mg/L)	**EC_{50} (mg/L)**
methyl isothiocyanate	>10	7.86 ± 1.57
2,5-thiophenenedicarboxaldehyde	1.20 ± 0.15	1.13 ± 0,36
2,5-thiophenedinitrile	>250	>250
2-pyridinecarboxaldehyde[1]	25.0 ± 2.4	3.60 ± 1.13
3-pyridinecarboxaldehyde	>250	>10
2-thiophenenecarboxaldehyde[1]	105 ± 10	14.08 ± 1.86
2-thiophenecarboxaldehyde oxime	>250	>250
2-thiophenecarbonitrile	>100	>100
5-formylthiophene-2-carbonitrile	>250	>250
indole-3-carbonitrile	>250	68.9 ± 14.1
indole-3-carboxaldehyde	>250	301 ± 33
1H-indole-2,3-dione	>250	>250
pyrrole-2-carboxaldehyde	>1000	392 ± 32
2-pyrrolidone	>250	>250

N-methyl-2-pyrrolecarboxaldehyde	>1000	>1000
furfural	11.40 ± 1.25	8.50 ± 0.89
fosthiazate	3.30 ± 0.95	0.40 ± 0.30

[A1] These compounds showed a strong

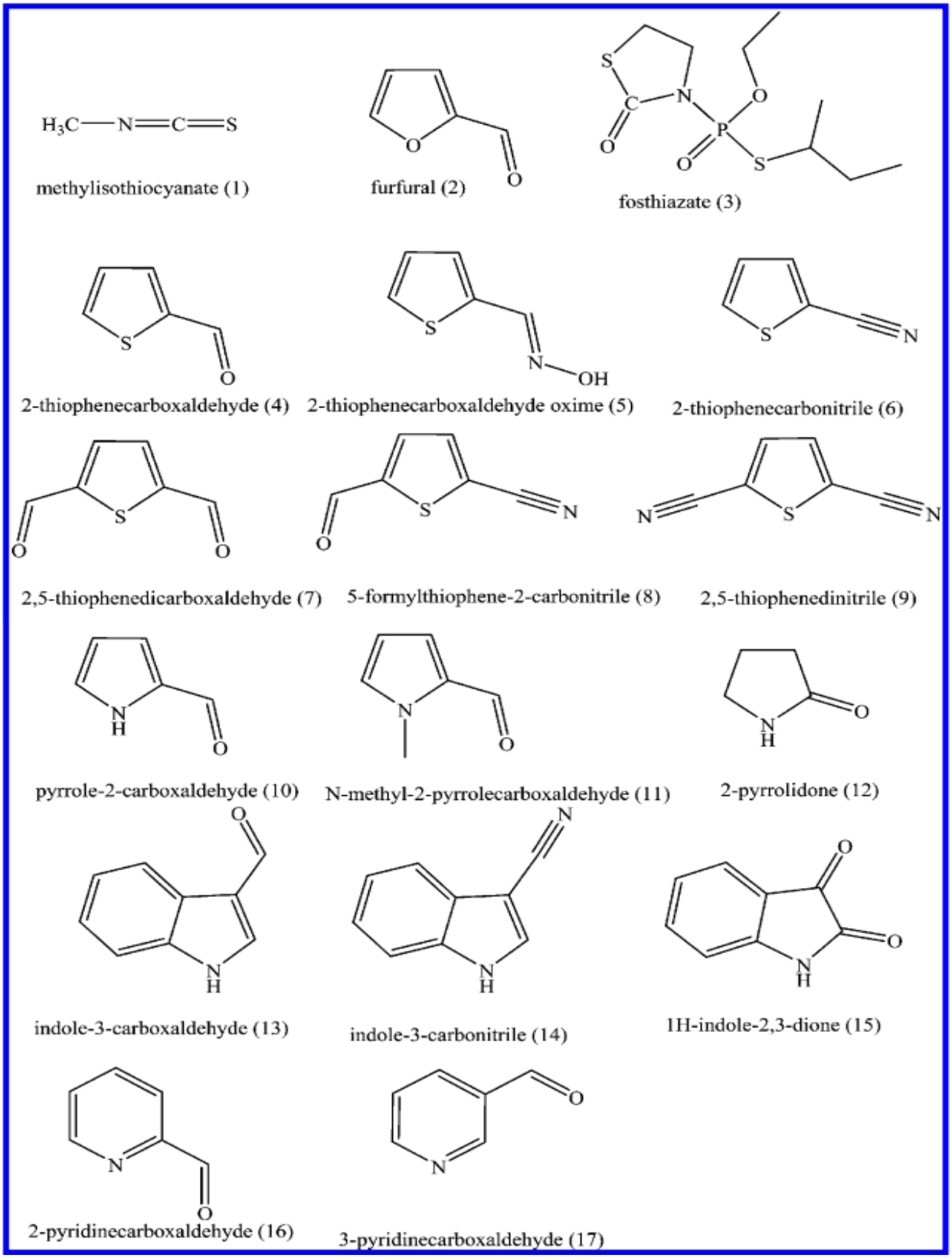


Figure 14: Chemical structure of the tested compounds.

Moreover, 2-pyridinecarboxaldehyde was very active, showing an EC50/1d of 3.60 mg/L; conversely, moving the carbonyl group from the 2 to the 3 position, we did not measured any activity. We also observed a toxin-mediated fast nematode paralytic killing that correlates with the concentration of heteroaromatic aldehydes. As previous reported, there is difference between the form of ded nematodes treated with aldehydes and muscles blockers. In particular, from a structural point of view, the most active compounds, thiophene-2,5-dicarbaldehyde and 2-pyridinecarbaldehyde, share some interesting features. In fact, they possess high aromatic scaffolds and both support two hydrogen-bond acceptors that seem to be placed in a suitable position. Many reasons might be adduced for the lack of activity of the other compounds showed in **Figure 14**. In particular, different bond geometry, weaker hydrogen bond acceptor strength, and different oxidation state together with a reduced electrophilicity of the carbon atom might be involved. Besides, heteroaromatic aldehydes are relatively reactive compounds due to the presence of the carbonyl carbon atom, an electrophilic site that may easily react with primary amino and thiol groups, resulting in formation of Schiff bases and hemithioacetals, respectively. According to this chemical behavior, the cuticle damage that occurs when nematodes are treated with furfural at 100 mg/L might be adducted to a nucleophilic addition of the cuticle amino or thiol residues the carbonyl functionality of the aldehyde. As previously reported, this interaction leads to evident cuticle and internal damage followed by leakage of the internal fluid nematode material.[95] Similar nematode cuticle damage was reported for *Panagrellus redivivus* caused by a unique fungal structure on the vegetative hyphae of *Coprinus comatus*.[96,97] Additionally, Niu et al. reported that aurovertin-type metabolites from the fungal strain *Pochonia chlamydosporia* lead to destruction of the internal structure of the nematode *P. redivivus*.[98]

Aurovertin-type metabolites produced by the strain of *C. arbuscolo* are regarded as potent inhibitors of ATP-synthesis and ATP-hydrolysis uncoupling oxidative phosporylation.[99]

IV. Nematicidal Activity of Allylisothiocyanate from Horseradish (*Armoracia rusticana*) Roots against *Meloidogyne incognita*:

IV. 1. *Armoracia rusticana*:

Horseradish (*Armoracia rusticana*) is a perennial crop, probably native to southeastern Europe and western Asia, belonging to the genus Armoracia of the Brassicaceae family. Horseradish is perennial in hardiness zones 2–9 and can be grown as an annual in other zones, although not as successfully as in zones with both a long growing season and winter temperatures cold enough to ensure plant dormancy. After the first frost in the autumn kills the leaves, the root is dug and divided. The main root is harvested and one or more large offshoots of the main root are replanted to produce next year's crop. Roots are popularly used as a pungent spice and as an indispensable material for producing horseradish paste and pungent sauces, and it is a common substitute of wasabi in Japanese traditional sushi[100] **(Figur 15)**. It is also used to treat nonspecific urinary tract infections and seems to inhibit the growth of colon and lung cancer cells.[101,102] This plant contains several isothiocyanates, which are known for their antibacterial, antifungal, and insecticidal activities.[103] As previously reported, Glucosinolates are a class of secondary compounds widely distributed throughout the Cruciferae. Glucosinolates are D-thioglucosides distinguished from one another by the differences in their organic side chains (R groups), according to which glucosinolates are grouped as either aliphatic, aromatic, or indole forms. They occur in all plant tissues and degrade via enzymatic hydrolysis. Tissue damage brings glucosinolates in contact with myrosinase and as a result, glucose and sulfates are released along with several toxic and pungent products, such as isothiocyanates, nitriles, and oxazolidine-thiones, depending on the parent glucosinolate, pH, and other factors.[104] Interestingly, the suppressive nematicidal effect of soil-incorporated Brassicaceae plant parts containing glucosinolates has been known since 1951, and today it is known as “biofumigation”.

Figure 15: *Armoracia rusticana* roots

IV. 2. Materials and Methods:

IV. 2. a. Chemicals:

Allylisothiocyanate, benzylisothiocyanate, methylisothiocyanate, phenylisothiocyanate and fosthiazate were obtained from Sigma-Aldrich (Milano, Italy), methanol, formic acid, and water were of high-performance liquid chromatography (HPLC)-grade.

IV. 2. b. Plant material and extraction:

Roots of *A. rusticana* (5 kg) were collected from Ferrara (Emilia-Romagna, Italy) in June 2012. The extraction was made as previously mentioned using a mixture of methanol and water (1:1, w/v) for 2 days then was analyzed by GC-MS and LC-MS. The moisture was found to be 69.21%.

IV. 2. c. GC-MS Analysis:

Roots Extract. Chromatographic separation and identification of components of methanol aqueous extracts of *A. rusticana* were performed on a gas chromatograph Varian model 3800 equipped with a Varian 7800 autosampler, a split/splitless injector Varian 1079, and an ion

trap mass detector (ITMS) 2000. The analytical column was a Varian VF5ms (30 m × 0.25 mm i.d. × 0.25 μm film thickness) (Varian, Milan, Italy). Helium was the carrier gas at 1 mL/min. A 1 μL sample, previously dehydrated with sodium sulfate, was injected in splitless mode with a purge valve on at 1 min. The injector temperature was set at 200 °C. The mass spectrometer was calibrated weekly, following the autotune test of the software (Saturn GC-MS Workstation 5.41). The mass spectrometer detector was operated in electron ionization positive mode. Trap, manifold, and transfer line temperatures were at 200, 80, and 200 °C, respectively. The oven was programmed as follows: 50 °C (1 min), raised to 100 °C (5 °C/min), and held for 1 min, then raised to 180 °C (20 °C/ min), and held for 4 min. Qualitative analysis was performed in the scan mode (50-550 amu). Peak identification was made comparing full mass spectra and retention times from authentic standards and the NIST MS Spectra Library (The NIST Mass Spectral Search Program for the NIST\EPA\NIH mass Spectral Library, version 2.0, build 12/2000).

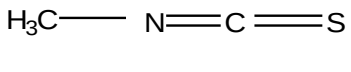
Isothiocyanates Degradation Studies in Soils. A soil sample (20 kg) was collected from an open field in Uta, Sardinia (Italy) in 2012 from the top 20 cm. Macroresidual materials, macro faunal remains, and stones were accurately removed, and larger soil aggregates were manually fragmented into smaller ones, prior to the subsequent fractionation procedure. Soils were air-dried and sieved <2 mm, separated into pots of 200 g, divided into two categories, autoclaved (11 min at 121 °C, 15 psi) to destroy microbes (such as bacteria, actinomycetes, fungi, and protozoa), and non autoclaved, and then spiked with 100 μL of 100 g/L of isothiocyanates mixture and kept in the dark at room temperature. At 0, 10, 20, 30, 60, 120, and 240 min of incubation, 200 g of soil was extracted with 200 mL of methanol, sonicated for 15 min, filtered through a Whatman no. 40 filter paper, and centrifuged for 15 min at 13000 rpm and then injected for GC-MS analysis. The analysis was performed as in the selection ion storage (SIS) mode using the following ions: m/z 99 for allylisothiocyanate,

m/z 135 for phenylisothiocyanate, and m/z 149 for benzylisothiocyanate, integrating the peak area of the GC-MS chromatograms versus concentration. Four replicates were analyzed.

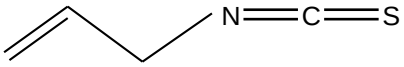
IV. 2. d. LC-MS/MS Analysis:

A Varian 1200 L triple-quadrupole tandem mass spectrometer (Palo Alto, CA) coupled with a ProStar 410 autosampler and two ProStar 210 pumps and a 1200 L triple-quadrupole mass spectrometer was used with an electrospray ionization (ESI) source. The Varian MS workstation, version 6.7, software was used for data acquisition and processing. Chromatographic separation was performed on Zorbax Column Synergi 4μm MAX-RP 80A (150 × 4.6 mm) (Phenomenex). The mobile phase consisted of (A) double distilled water and (B) methanol containing 0.1% formic acid. The solvent gradient started at 10% of B reaching 100% in 20 min and 100% in 25 min followed by post-time isocratic conditions for 5 min at 10% of B before the next injection. The mobile phase, previously degassed with high-purity helium, was pumped at a flow rate of 0.3 mL/min, and the injection volume was 10 μL. ESI was operated in negative ion mode. The electrospray capillary potential was set to -40 V, the needle at -4500 V, and the shield at -600 V. Nitrogen at 48 mTorr and 41°C was used as a drying gas for solvent evaporation. The atmospheric pressure ionization (API) housing was kept at 50 °C. Parent compounds were subjected to collision-induced dissociation using argon at 2.40 mTorr in multiple reaction monitoring (MRM) in the negative mode. **Table 10** reports the observed mass transitions and collision energy used for quantitation of different phenolics. The scan time was 1 s, and the detector multiplier voltage was set to 2000 V, with an isolation width of m/z 1 for quadrupole 1 and m/z 1.9 for quadrupole 3. The extract as well as Allylisothiocyanate, other aliphatic and aromatic isothiocyanates such as benzylisothiocyanate, methylisothiocyanate, and phenylisothiocyanate **(Figure 16)** were tested against J2 at a dose range of 1.0−1 000 mg /L using the organophosphorus fosthiazate

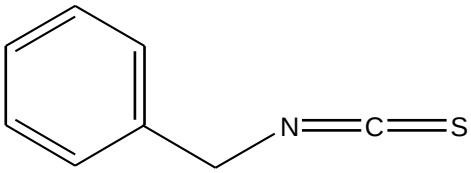
as a chemical control. In the same experiment, malic acid, gallic acid, ferulic acid, and (−)epigallocatechin-3-gallate, found by LC-MS/MS in the extract were tested against J2.



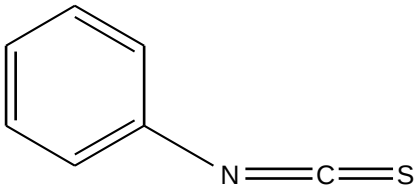
Methylisothiocyanate



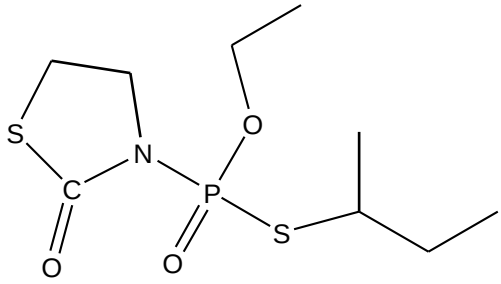
Allylisothiocynate



Benzylisothiocyanate



Phenylisothiocyanate



Fosthiazate

Figure 16: Chemical structures of tested compounds.

IV. 3. Results:

When the extract was tested against *M. incognita*, a clear dose-response relationship was established and significant paralysis/death of J2 was evident after 3 days of exposure with a calculated EC50/3d value of 250.7 ± 45.8 mg/L. By GC-MS analysis, we were able to quantify the most abundant plant metabolite, allylisothiocyanate, at a concentration of

10893 ± 7810 μg/g (30.1%), while the identification, by the NIST library of other constituents of the extract, was unsuccessful. Allylisothiocyanate applied against J2 was active with an EC50/1d of 6.6 ± 3.4 mg/L. This is the first report of the nematicidal activity of allylisothiocyanate as a constituent of *A. rusticana* against *M. incognita*. Zasada et al. tested this compound against *M. javanica* and found an LC50 of 10 mg/L.[105] Allylisothiocyanate was also found in mustard and is a practical alternative to fenamiphos, one of the most widely used nematicides in the turfgrass industry.[106]

Table 10: Multiple Reaction Monitoring Transitions (LC-MS/MS) and Phenolic Compounds in *Armoracia rusticana*

			first transition		second transition			
compd	MW	precursor mass (m/z)	mass (m/z)	CE (V)	mass (m/z)	CE (V)	R_t (min)	concn ($\mu g/g$)
malic acid	134	$[M - H]^-$	133.0→71.0	(13.5)	133.0→114.8	(9.0)	8.47	307 ± 245
gallic acid	170	$[M - H]^-$	169.0→124.8	(12.5)			18.04	1.89 ± 0.62
ferulic acid	194	$[M - H]^-$	193.0→177.7	(16.0)	193.0→133.8	(18.0)	18.88	35.1 ± 20.3
(−)epigallocatechin-3-gallate	458	$[M - H]^-$	457→168.7	(15.5)	457→304.9	(17.5)	20.56	7.82 ± 5.43
myricitrin	464	$[M - H]^-$	463→286.9	(42)	463→317.0	(21.0)	22.85	5.09 ± 1.95

Table 11: EC 50 Values (mg/L) (± SD, n = 4) of Pure Compounds against *M. incognita* at 1 h and 1 day after Treatment

compound	1h, EC_{50} (mg/L)	1d, EC_{50} (mg/L)
allylisothiocyanate[a]	52.6 ± 45.6	6.6 ± 3.4
benzylisothiocyanate[a]	11.7 ± 7.2	1.9 ± 1.1
methylisothiocyanate	54.4 ± 16.2	7.9 ± 1.6
phenylisothiocyanate	>1000	>1000
malic acid	>1000	>1000
gallic acid	>1000	>1000
ferulic acid	>1000	>1000
myricitrin	NT	NT
(-)epigallocatechin-3-gallate	>1000	>1000
fosthiazate	3.3 ± 1.0	0.40 ± 0.3

We then tested other isothiocyanates with structure similarities to the allylisothiocyanate, and the respective EC50 values are presented in **Table 11**. Slight structural differences can confer important different nematicidal effects, confirming that biological activity is a function not only of the concentration of the product but also of the chemical properties of the alkyl side chain.[107] When the double bound of the alkyl side chain was substituted by a benzene moiety as in the case of benzenisothiocyanate, the compound was three times more active. We also observed that J2 treated with different isothiocyanates were paralyzed or died in a straight shape in contrast of those treated with the organophosphorous fosthiazate as previously reported. As electrophiles, isothiocyanates and heteroaromatic aldehydes are capable of reacting with sulfhydryl groups and binding to protein amino and thiols groups by interaction

with cystein, and these modifications may alter protein functions.[108,109] Malic acid, gallic acid, ferulic acid, and (−)epigallocatechin-3-gallate, determined by LC-MS/MS in the extract, were not active at the doses 100−1000 mg/L as previously reported by Aoudia et al.[110]

Because of the high volatility of isothiocyanates, we then tested the persistence of structurally related isothiocyanates in soils, and no significant differences on half-life time in autoclaved and nonautoclaved soil by GC-MS analysis were detected; however, high volatility is noted **(Figure 17)**. This fact shows that isothiocyanate degradation in soil is not due to biological factors. In all cases, allylisothiocyanate was the most volatile compound with a half-life time lower than 10 min followed by phenylisothiocyanate and benzylisothiocyanate. Our results are similar to those by Borek et al. who saw no effect on the transformation of allylisothiocyanate in autoclaved soil.[111]

The degradation of chemicals depends mostly on soil composition, moisture, and temperature. Physical and chemical properties of the soil used in this study are shown in **Table 12**.

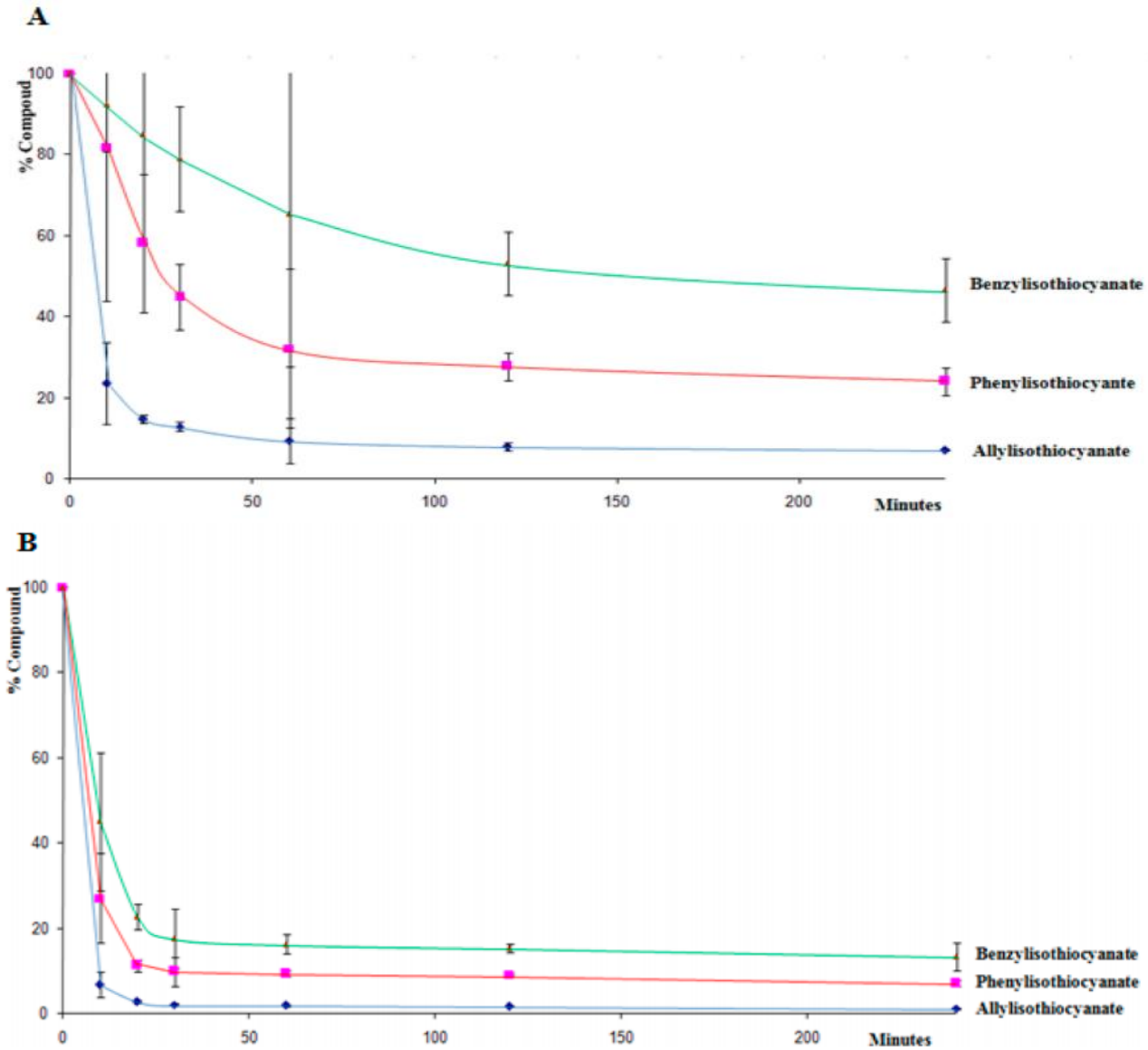


Figure 17: Isothiocyanate degradation profile in soil: (A) non-autoclaved soil and (B) autoclaved soil.

Table 12: Physicochemical Properties of Soil

parameter	g/kg
sand	442
silt	196
clay	362
total organic substance	26.2
total nitrogen	1.56
pH in H_2O	8.25

Faster rates of allylisothiocyanate disappearance occurred in soils with high organic carbon contents and total nitrogen concentrations, reduced soil moisture, and high temperature.[111]

In both sterile and nonsterile soils occurred an initial fast decrease rate followed by a much slower decline, which clearly indicates the rather complex chemical process of isothiocyanate degradation in soil. This initial rate was most likely a combination of volatilization, irreversible partitioning into the organic matter of the soil, and reactivity with nucleophilic groups.[112] This was supported by the observation that it was possible, at the first sampling time, to recover more benzylisothiocyanate than phenylisothiocyanate and allylisothiocyanate taking into account that benzylisothiocyanate is not very reactive. Autoclaving the soil did not affect the half-life probably due to the inhibiting effect of isothiocyanates on bacterial growth, just as reported by Klose et al. 2006,[113] or also Due to the fast volatility of isothiocyanates not permitting degradation by bacteria.

V. Nematotoxic Phenolic Compounds from *Melia azedarach* Against *Meloidogyne incognita*:

V. 1. *Melia azedarach*

Melia azedarach, commonly known by many names, including white cedar, chinaberry tree, bead-tree, Cape lilac, syringe berrytree, Persian lilac, and Indian lilac, is a species of deciduous tree in the mahogany family, *Meliaceae*, that is native to Indomalaya and Australasia.[114]

The adult tree has a rounded crown, and commonly measures attains a height of 7-12 metres, however in exceptional circumstances *M. azedarach* can attain a height of 45 metres.[115] The flowers are small and fragrant, with five pale purple or lilac petals, growing in clusters. The fruit is a drupe, marble-sized, light yellow at maturity, hanging on the tree all winter, and gradually becoming wrinkled and almost white. The leaves are up to 50 cm long, alternate, long-petioled, two or three times compound (odd-pinnate); the leaflets are dark green above and lighter green below, with serrate margins **(Figure 18)**. Leaves are used in leprosy, scrofula, anthelmintic, antilithic, diuretic, deobstruent, and resolvent. Roots are effectively used as resolvent and deobstruent. Seed oil is the most active medicinal product of the plant and used as an antiseptic for sores and ulcers that show no tendency to heal. It is also used for rheumatism and skin diseases such as ringworm and scabies. Internally, the oil is useful in malaria fever and leprosy.[116] Seeds of *M. azedarach* L. have been scientifically reported to exert insecticidal and antifeedant activities.[117-118] Interestingly, the extract of *M. azedarach* was found to contain higher amounts of phenolic compounds in comparison to the species *Azadirachta indica* and, thus, to exhibit significant scavenging property mainly due to the presence of constituents bearing hydroxyl groups.[119]

Phenolic acids are known to exhibit nematicidal properties[120] while the nematode infection, in turn, influences the levels of phenolic compounds in tomato plants as a result to the induction of abiotic stress.[121]

Figure 18: Melia tree

V. 2. Materials and Methods:

V. 2. a. Chemicals:

Analytical standards of caffeic acid, ferulic acid, gallic acid, protocatechin, vanillic acid, chlorogenic acid, β-naphtoic acid, nicotinic acid, salicylic acid, benzoic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid, malic acid, epicatechin, rutin, and fosthiazate were purchased from Sigma-Aldrich, Italy (purity ≥98−99%). The standards were used for component identification analysis and for nematodes bioassays. All solvents and reagents were of analytical grade.

V. 2. b. Extraction of Plant Materials.

Ripe fruits (4 kg) of *M. azedarach* were collected at Cagliari, Sardinia, Italy, and Targa ouzemour, Bejaia, Algeria. The fruits were kept in the dark at room temperature for 30 days. Fruits were then separated in parts (pulp, seeds, stone, and kernel) and immediately analyzed. Voucher specimens were deposited in the Department of Life and Environmental Sciences (University of Cagliari, Italy) for species identification. Then Dried powdered fruit parts (pulp, seeds, stone, and kernel) of the Algerian and Italian *M. azedarach* specimens were extracted with water (1:5, w/v) and sonicated for 30 min, filtered through a Whatman no. 40, and finally centrifuged for 10 min at 11000 rpm. Plant materials were also dried at 105 °C for 24 h, and the moisture content was calculated. The extraction yield was determined on average over three replicates.

V. 2. c. Apparatus and Chromatography.

HPLC-MS/MS Analysis.

For the identification and quantitation of the phenolic compounds in the nematicidal extracts against J2 of *M. incognita,* LC-MS/MS was used in the same conditions previously reported.

HPLC-DAD Analysis.

For confirmation, an Agilent Technologies (Waldbronn, Germany) model 1100 high performance liquid chromatograph was used fitted with a diode array detector (DAD) mode. An analytical column Waters Spherisorb 5 μm ODS2 (4.6 mm×250 mm Analytical Cartridge) (Milford, MA) was employed. For HPLC analysis, an aliquot (100 μL) was injected into the column and eluted at 40 °C. For the analytical separation, the gradient profile of the mobile phase consisting of (A) acetonitrile and (B) 0.22 M H_3PO_4 (10:90, v/v) at the constant flow of 1 mL/min was as follows: initial start at 10% of A reaching 100% in 20 min and hold to

20 min. Before the next injection, the HPLC system had to be stabilized for 10 min with acetonitrile/aqueous 0.22 M H_3PO_4 (10:90, v/v). Detection was carried out at wavelengths of 210, 280, 313, and 360 nm. Analysis was performed three times (n = 3).

V. 3. Results:

HPLC-DAD and triple quadrupole mass spectrometry detectors are the elective techniques for the chemical characterization and quantitation of phenolic compounds in botanical extracts. In the frame of our continuous search for nematicidal components of plant origin, we studied the water extracts of chinaberry. The activity of *M. azedarach* extracts against *M. incognita* was found only in the pulp; therefore, this was the only fruit part that we decided to study for the chemical composition. Specifically, we performed the chemical characterization of the Algerian pulp water extract (APWE) and Italian pulp water extract (IPWE) by means of HPLC-DAD and HPLC-MS/MS techniques. The most abundant phenolic compounds present in the IPWE were *p*-coumaric, malic, nicotinic, vanillic, and ferulic acids, while in the APWE extracts, we found *p*-coumaric, vanillic, malic, and chlorogenic acids. In **Table 13**, we report the UV elution time of phenolic compounds present in the extracts together with their UV max absorbances, while **Table 14** reports the observed mass transitions and collision energy used for quantitation of different phenolics. The only extract exhibiting substantial nematicidal activity was the water pulp extract (IPWE) of the Italian *M. azedarach*, probably due to the difference in the chemical composition based on geographical distribution. Specifically, the IPWE EC50 value calculated after 48h of nematodes immersion in test solutions was 955 μg/mL (±SD 300.1), while J2 immersion in 1540 μg/mL for 1 day induced 100% paralysis. Contrary to the Italian *M. azedarach*, the Algerian was not found nematicidal at the dose range of 100-2500 μg/ml. When pure phenolic acids were tested on J2, it was shown that the most nematicidal was salicylic acid followed by benzoic, *p*-coumaric, *p* -hydroxybenzoic, and nicotinic acid (EC50/1d=379, 501, 840, 871,

and 1732 μg/mL, respectively) (**Table 15**). Caffeic, ferulic, gallic, vanillic, chlorogenic, b-naphtoic, malic acid, protocatechin, epicatechin, and rutin were not active at the doses 100 −1000 μ g/mL. *p*-coumaric was present in IPWE and APWE extracts at the concentrations of 79.9 and 0.52 μg/g, respectively, while *p*-hydroxybenzoic was found only in IPME (0.24 μg/g). *p*-coumaric and *p*-hydroxybenzoic EC50 values after 24 h of J2 immersion in test solutions were 840 and 871 μg/mL, respectively. Interestingly, salicylic acid paralyzed all nematodes after 1 h of immersion in 1000 μg/mL. Also, previously, it has been reported the strong nematicidal activity of salicylic acid as component of other nematicidal botanicals. Specifically, salicylic acid in nematodes infested tobacco cultivars, as a result to *Pseudomonas fluorescens* and *Trichoderma harzianum* applications in *M. incognita*, was found to decrease root galls and egg masses/root system.[122] According to Wuyts and co-workers, salicylic acid is a strong attractant for *M. incognita*, but it is also nematicidal (LC50 of 46 μg/mL) and an irreversible inhibitor of hatch.[123]

Table 13. Retention Time and UV max Absorbances of the Phenolic Compounds Identified in the APWE and the IPWE by HPLC Analyses

compd	t_R (min)	UV max absorbance
nicotinic acid	3.639	210, 280
malic acid		
p-coumaric acid	9.119	210, 280, 313
vanillic acid	7.273	210
gallic acid	3.219	210
caffeic acid	6.659	313
ferulic acid	10.527	313
Protocatechin	4.437	210
p-hydroxybenzoic acid	6.269	210
chlorogenic acid	3.921	313
rutin	12.519	210

Table 14. MRM Transitions and Content of Phenolic Compounds in *M. azedarach*

compd	MW	precursor mass (m/z)	first transition		second transition		R_t (min)	concn (μg/g ± SD; n = 3)	
			mass (m/z)	CE (V)	mass (m/z)	CE (V)		APWE	IPWE
nicotinic acid	123	$[M-H]^-$	121.0 → 77.9	13.0			8.47	1.57 ± 0.51	
malic acid	134	$[M-H]^-$	133.0 → 71.0	13.5	133.0 → 114.8	9.0	10.86	91.1 ± 33.7	1170 ± 345
p-coumaric acid	164	$[M-H]^-$	163.0 → 92.9	34.0	163.0 → 118.9	16.0	19.28	0.52 ± 0.22	79.9 ± 41.7
vanillic acid	168	$[M-H]^-$	167.0 → 107.8	24.0	167.0 → 122.8	14.0	18.04	17.0 ± 5.9	56.2 ± 20.0
gallic acid	170	$[M-H]^-$	169.0 → 124.8	12.5			15.66	0.53 ± 0.21	0.26 ± 0.16
caffeic acid	180	$[M-H]^-$	179.0 → 133.7	26.0	179.0 → 134.8	16.0	19.09	29.5 ± 7.89	36.6 ± 8.4
ferulic acid	194	$[M-H]^-$	193.0 → 177.7	16.0	193.0 → 133.8	18.0	19.51	87.4 ± 22.0	278 ± 76
3,4-dihydroxybenzoic acid	154	$[M-H]^-$	153.0 → 107.8	26.0	153.0 → 108.9	16.0	16.26	1.16 ± 0.58	3.70 ± 1.49
p-hydroxybenzoic acid	138	$[M-H]^-$	137.0 → 92.9	13.5			20.30		0.24 ± 0.10
salicylic acid	138	$[M-H]^-$	137.0 → 92.9	14.5			20.20		0.91 ± 0.55
rutin	610	$[M-H]^-$	609.0 → 270.6	46.5	609.0 → 299.6	35.5	21.72	8.53 ± 3.99	136 ± 33
isoquercitin	464	$[M-H]^-$	463.0 → 270.7	41.5	463.0 → 300.9	19.0	21.71		1.81 ± 0.46

Salicylic acid is determined as an elicitor of systemic acquired resistance, inhibiting catalase activity. Salicylic acid together with nicotinamide, 2-chloronicotinic acid, 5-nitrosalicylic acid, 4-chlorosalicylic acid, DL-2 aminobutyric acid, 2-aminobutyric acid, O-acetylsalicylic acid, and 4-amino salicylic acid, used as soil drench induced reduction in root-knot severity, suggesting a strong possibility of the use of these activators in integrated management.[124] Moreover, salicylic acid was found to increase the terpenoid aldehyde content of cotton roots, while in plants inoculated with root knot nematodes and treated with salicylic acid, additional amounts of root gossypol and hemigossypolone were induced.[125] Similarly, coumaric and *p*-hydroxybenzoic acids have already been reported of significant nematicidal activity against *Meloidogyne* spp. Specifically, caffeic, ferulic, coumaric, benzoic, vanillic, chlorogenic, and hydroxybenzoic acid found in the aqueous extracts of dry and fresh leaves of *Eucalyptus citriodora* extracts were found to exhibit significant nematicidal activities.[126] *p*-Hydroxybenzoic acid, vanillic acid, caffeic acid, ferulic acid, and a quercetin glycoside,

7-glucoside, were found to have a considerable nematicidal activity against as contained in root leachate of *Lantana camara*.[127] Coumaric, ferulic, salicylic, and benzoic acids, used as soil drench in propolis extract, decreased the juvenile *Meloidogyne* sp. population density and the number of root galls in bean plants when tested at 1000 mg/L.[128] According to our results, the EC50 values of benzoic and salicylic acid were comparable (501 and 379 μg/mL, respectively), while the introduction of an hydroxyl group in the para position **(Figure 19)** reduced by a half the activity against J2. *p*-coumaric acid showed an EC50 value of 840 μg/mL, and when caffeic acid was tested for comparison, there was not observed any activity at 1000 μg/ml. The difference in the chemical structure of the nematicidal phenolic compounds probably implies differences in the modes of action against nematodes, resulting in decreased probability of resistance development. Furfural was found to exhibit an EC50 value of 8.5 μg/mL after 24 h of J2 immersion in test solutions, while organic acids EC50 values ranged from 38.3 to 45.7 μg/mL, with acetic acid being the most active. In **Figure 20** are presented the damages on J2 body after immersion in acetic acid solutions. It is evident the removal of the cuticle from the internal organs of the nematode's body, probably due to the osmoregulation disruption and subsequent fluid accumulation. All other experimental treatments, including phenolics treatments, paralyzed J2 but did not induce such damages on juveniles body, apart for furfural.

Table 15. EC50 and SD Values of Individual Compound s against *M. incognita* Calculated at 24 h of I mmersion in Test Solutions

compd	24 h of J2 immersion in test solutions: EC_{50} µg/mL (SD)	compd not present in *M. azedarach*	24 h of J2 immersion in test solutions: EC_{50} µg/mL (SD)
nicotinic acid	1732 ± 730	benzoic acid	501 ± 158
salicylic acid	379 ± 96	malic acid	>1000
p-hydroxybenzoic acid	871 ± 222	vanillic acid	>1000
p-coumaric acid	840 ± 196	gallic acid	>1000
caffeic acid	>1000	*β*-naphtoic acid	>1000
protocatechin/ 3,4-dihydroxybenzoic acid	>1000	ferulic acid	>1000
epicatechin	>1000	chlorogenic acid	>1000
rutin	>1000	fosthiazate	0.4 ± 0.93
furfurale[b]	8.5 ± 2.29	acetic acid[b]	38.3 ± 22.0
5-hydroxymethyl-furfurale[b]	45.7 ± 11.8	butyric acid[b]	40.7 ± 21.3
furfurol[b]	41.2 ± 10.1	hexanoic acid[b]	41.1 ± 20.0

[b]Data reported in Ntalli et al. (2010).[129]

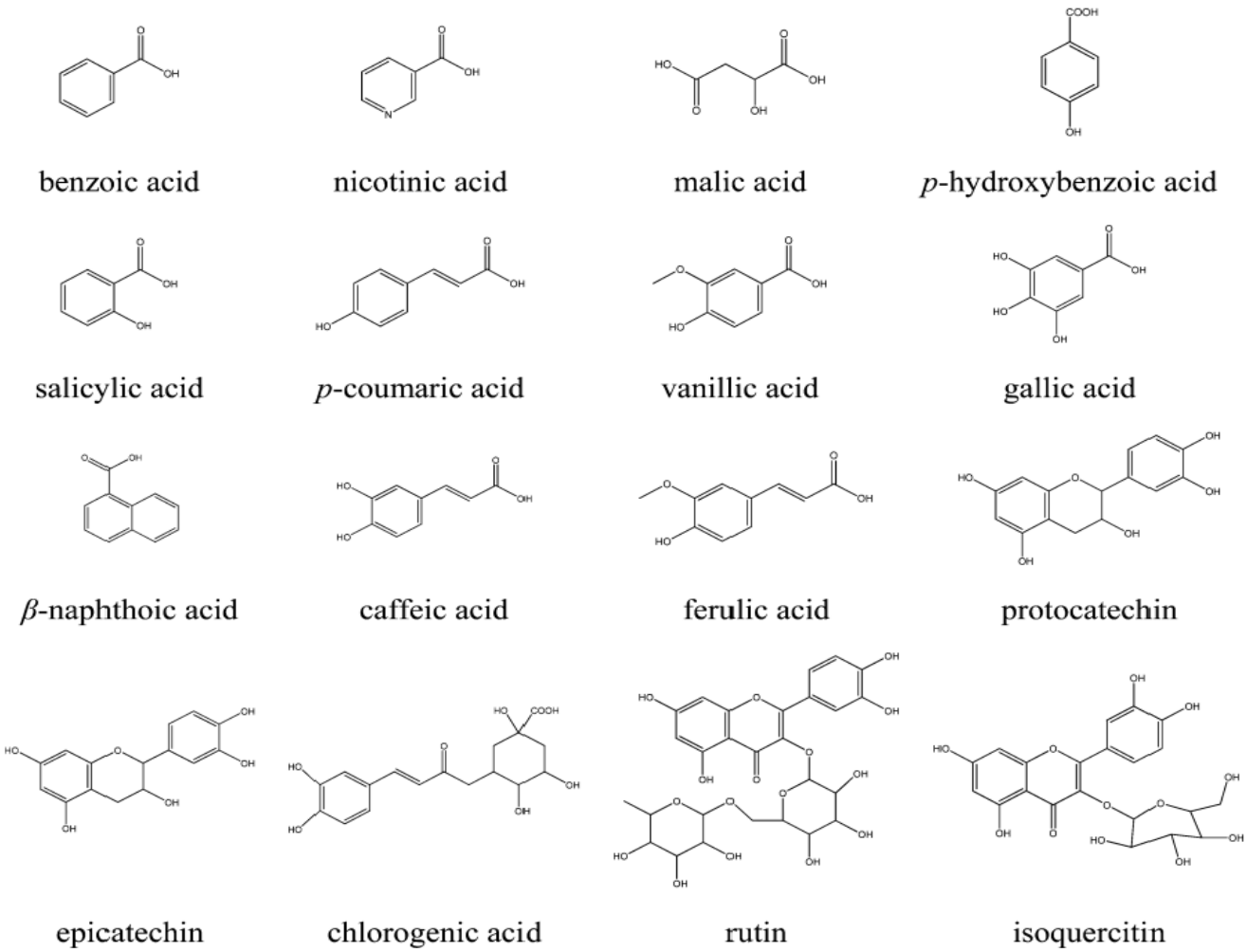


Figure 19: Chemical structures of phenolic acids used for bioassays against *M. incognita* J2.

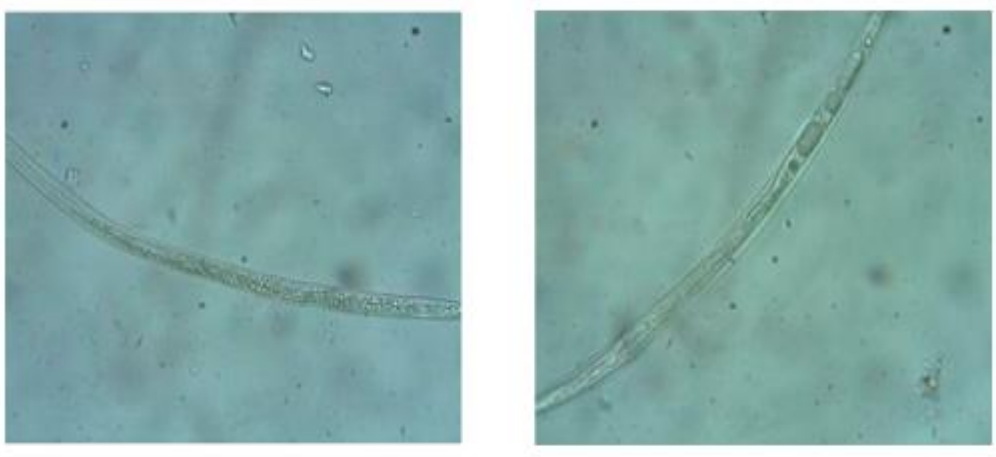


Figure 20. Microscope images from immersion treatment of J2 in (left) water and (right) acetic acid at 100 µg/mL.

CHAPTER II

ANTIMICROBIAL ACTIVITY OF POLYPHENOLS:

Inhibitory Effect of Carob (*Ceratonia siliqua*) Leaves Methanolic Extract on *Listeria monocytogenes*:

I. *Ceratonia siliqua:*

Carob (*Ceratonia siliqua*) **(Figure 21)** is a tree widely grown in the Mediterranean regions. It belongs to the Caesalpinaceae, a subfamily of Leguminoseae family (Fabaceae). It grows well in warm temperate and subtropical areas, and tolerates hot and humid coastal areas. As xerophytic (drought-resistant), this specie has developed special mechanism to survive in the Mediterranean conditions, and its introduction in semi-arid lands may help to prevent the disruption of the equilibrium of those fragile ecosystems.[130] Carob is a desired ingredient in the kitchens all over the Mediterranean basin. Consumed since ancient times, this plant is also known as Saint John's Bread or locust bean. The dried carob fruit is traditionally consumed on Jewish holidays and the juice is taken by Muslims, during the Islamic month of Ramadan. It is also used as a substitute in making various products such as baked goods, bars, snacks, cereal, dairy products, cocoa-containing products and beverages. The gum is employed in a wide range of products in the food industry; such as ice cream, baby foods and pet foods.[131]

It is also found the benefits of using Carob pod as a substitute for Chocolate. In fact, it have a high level of calcium and has neither the addictive qualities of chocolate, nor the caffeine.[132]

Carob pods are found to contain proteins, fat, carbohydrates, polyphenols, and tannins.[133] The bark and leaves of this tree are used in Turkish folk medicine as an antidiarrheal and diuretic.[134] The leaves are also used for their antioxidant activities.[135] The fruits are traditionally used as an antitussive and against warts.[136] The gum is a galactomannan, a valuable natural food additive for products such as ice cream, sweets, and soups. Carob is also used in the textile and cosmetics industries.[137]

Figure 21: Carob tree

II. Materials and Methods:

II. a. Chemicals:

Standards of gallic acid, (−)-epigallocatichin-3-gallate, myricitrin, isoquercitin, malic acid, catechin, chlorogenic acid, and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (Milano, Italy), and L-(−)-proline (Carlo Erba Milano, Italy), methanol, phosphoric acid, acetonitrile, formic acid, and water were of high-performance liquid chromatography (HPLC)-grade.

II. b. Plant material, extraction and analysis:

Leaves of *C. siliqua* were collected from Capoterra (Sardinia, Italy) in March, 2012, and were dried in the absence of light at room temperature. Then, the methanol extraction was made as previously reported and phenolic compounds were determined using previously described HPLC-DAD and HPLC -ESI-MS/MS techniques.[111]

II. c. Microbiological Assay:

MIC determination.

The antimicrobial activity of carob leaves methanolic extract was studied. The experiments were carried out using a set of microorganisms: *Escherichia coli* (ATCC 25922), *Salmonella typhimurium* (ATCC 14024), and *Pseudomonas aeruginosa* (ATCC 9027) strains for Gram (−), *L. monocytogenes* (ATCC 35152) and *S. aureus* (ATCC 25932) strains for Gram(+), and *Aspergillus niger* (ATCC 16404), *Fusarium oxysporum*, *Fusarium graminearum*, and *Penecillium* spp. as fungal strains. We also used *L. monocytogenes* strains isolated in our laboratory for this study. For each of the above-mentioned strains, the minimum inhibitory concentration (MIC) was determined using the broth microdilution method. Stock standard phenolics solutions at 1% (v/v) in DMSO were prepared. Working solutions were prepared by dilution in microplates at concentrations between 3.5 and 900 µg/mL. The bacterial suspensions were added in the microwells at a concentration of 10^5 colony-forming units/mL (CFU/mL). The plates were incubated aerobically at 37 °C for 24 h. Bacterial growth was revealed by the presence of turbidity and a "pellet" on the well bottom. MICs were determined as the first well that did not produce a pellet. Inhibited microorganisms were then tested using standard disk[138] to determine the MBC (minimal bactericide concentration). The MBC is the lowest concentration of a substance with a bactericidal effect of 99.99%. The experimental determination of MBC consists of measuring the vitality percent (%) without visible growth. The same experiment is repeated with carob leaves compounds deduced by HPLC and liquid chromatography/mass spectrometry (LC-MS), first checked individually and then in polyphenolic association to look for synergic activities, and in the presence of proline (1 mM). For fungal strains, the tests were carried out by insemination, with mycelia fragments of 6 mm in diameter (10 days hold), in Petri dishes containing potato dextrose agar (PDA). After the addition of the extract (450 and 900 µg/mL),

the plates were sealed with para film and incubated in the dark at 22 °C. Control samples with the mycelia in PDA and distilled water were incubated under the same conditions. The effectiveness of the treatments was evaluated by measuring the average diametric growth of the colonies after 4, 8, and 12 days of the inoculum. The percentage of inhibition (I) was calculated according to the formula of Zygadlo and Guzman[139]

$$I\ (\%) = [(\ C - T\ )/\ C\ ]\ X\ 100$$

Where C = average diameter of fungi grown in PDA + water, and T = average diameter of fungi cultivated in PDA with the extract. When no mycelium growth was observed, the latter was transferred to a plate containing only PDA and incubated for 48 h, to determine if the inhibition was fungistatic or fungicide. All experiments were repeated three times to confirm the MIC. For the assessment of listerial growth with and without inhibitor (MECL), a suspension of 10^3 CFU/mL of the bacterium was made in which we added the desired concentration of MECL, and 100 μL was plated in agar ALOA (Microbiol-Cagliari). This action was repeated in different plates after dilutions every 3 h with reference to a control without extract. The bacterium was incubated overnight at 37 °C, and then, the number of survived bacteria was counted.

Stability and Heat Inactivation of Carob Leaves Extract.

To assess the e ffects of storage on the activity of carob leaves extract, aliquots were stored at different temperatures (−18, +4, and 37 °C and at room temperature) for 24 days. Afterward, the MIC was redetermined.

Effect of Carob Extract on Some Enzyme Activity.

The changes of different cellular enzymatic activities of treated or untreated bacteria, collected from the inhibition and the exponential growth phase culture, were evaluated using

the API-ZYM system (BioMér-ieux). The API-ZYM system is a set of ready for use small tubes, which serve for identification of microorganisms by a rapid miniaturized biochemical test. One hundred microliters was plated in agar TSYEA and incubated for 24 h at 37 °C, and then, a suspension of 10^5 CFU/mL was made. Each API-ZYM gallery was inoculated with 100 µL of this last and incubated overnight at 37 °C.

II. d. Statistical Analysis.

All tests of the assessment of listerial growth were run in triplicate and averaged. Means, standard errors, standard deviations, and degrees of significance (using Student's *t* test) were calculated from replicates within the experiments, and analyses were done using Microsoft Excel XP 2010. Differences of $P < 0.05$ were considered to be significant.

III. Results:

The phenolic compounds of carob leaves methanolic extract were analyzed by HPLC-DAD and HPLC-MS. The use of DAD allows not only the peak identification but also peak purity determination. The purity of all identified peaks in our experiments generally reached 1.00, and the corresponding chromatogram obtained at 210 nm is presented in **Figure 22.** These compounds were identified by comparison of the retention times and UV/visible spectra with the available standards corresponding peaks. Four of the 12 observed peaks matched with the standard compounds used in this work were attributed to gallic acid, (−)-epigallocatechin-3-gallate, myricitrin, and isoquercitin, which correspond to compounds found in carob leaves.[140] Performing mass spectrometric analysis in the negative ion mode resulted in mass chromatograms for deprotonated ions $[M- H]^-$ at m/z ratios similar to those obtained with authentic standards. The ESI-MS technique allowed confirmation of and completion of the identification of the phenolic compounds based on their specific and characteristic molecular ions described in the literature. With this technique, we observed

three different phenolic compounds **(Table 16)**. Using the well microdilution method, MECL showed a high antimicrobial activity against both reference strain *L. monocytogenes* (ATCC 35152) and isolated strains with MIC at 28.12 µg/mL **(Table 17)**. This is the first report on the antilisterial activity of carob leaves methanolic extract. According to the Table 16, the (−)-epigallocatechin-3-gallate represents 12.71% of all polyphenols present in MECL or 114.39 µg/mL, while it is active against *S. aureus* at 200 µg/ml.[141] We can explain this resistance by the phenomenon of antagonism or synergism between all polyphenols present in MECL. No inhibition zone was detected when the disk diffusion method was used. This can indicate that carob leaves methanolic extract has a bacteriostatic activity against this bacterium. Bacteria that grow around the disk are those that may metabolize polyphenols present in MECL.

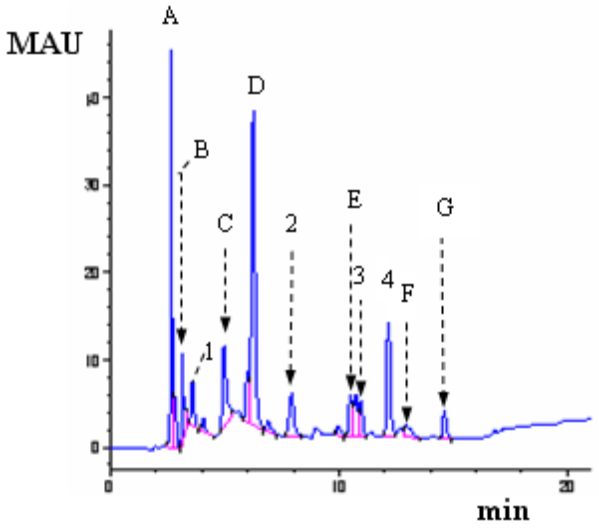


Figure 22: HPLC profile of carob leaves methanolic extract with detection at 210 nm. Identified peaks as compared to standard compounds: 1, gallic acid; 2, (-)epigallocatechin-3-gallate; 3,myricitrin; 4, isoquercitin. Unidentified peaks: A, B, C, D, E, F and G.

Table 16: Analyte HPLC-MS/MS (−) Transitions and Instrument Conditions

			first transition		second transition				
compd	MW	precursor mass (m/z)	mass (m/z)	CE (V)	mass (m/z)	CE (V)	R_t (min)	R^2	concn in MECL ($\mu g/g$) ± SD
gallic acid	170.1	$[M-H]^-$	169.1 → 124.8	12.5			10.517	0.9995	544 ± 310
(−)-epigallocatechin-3-gallate	458.37	$[M-H]^-$	457.4 → 304.9	17.5	457.9 → 168.7	15.5	13.012	0.9991	541 ± 135
isoquercitin	464.00	$[M-H]^-$	463.0 → 300.9	19.0	463.0 → 270.7	41.5	16.794	0.9998	131 ± 84
myricitrin	464.00	$[M-H]^-$	463.0 → 317.0	21.0	463.0 → 286.8	40.5	16.643	0.9996	1660 ± 999
chlorogenic acid	354.30	$[M-H]^-$	653.3 → 190.8	16.0			13.712	0.9979	9.70 ± 5.55
malic acid	134.00	$[M-H]^-$	133.0 → 071.0	13.5	133.0 → 114.8	09.0	07.848	0.9935	163 ± 53
catechin	290.00	$[M-H]^-$	289.0 → 202.8	18.0	289.0 → 244.7	14.0	14.642	0.9980	1205 ± 176

Table 17: Activity of Carob Leaves Methanol Extract against Bacterial and Fungal Strains[a]

Microorganisms	Technique	
	Microdilution method	Disk diffusion
	MIC (µg/mL)	Diameter of the inhibition zone (mm)
Gram (–)		
E. coli (ATCC 25922)	>900	NA
S. typhi (ATCC 14024)	>900	NA
P. aeruginosa (ATCC 9027)	>900	NA
Gram (+)		
L. monocytogenes (ATCC 35152)	28.12	NA
S. aureus (ATCC 25932)	>900	NA
Fungal strains		
A. niger (ATCC 16404)	-	NA
F. oxysporum	-	NA
F. graminearum	-	NA
Penecillium spp	-	NA

NA: Not active, -: not tested

To more specifically determine which components have inhibitory effects, pure phenolic compounds were examined. Results of MIC are presented in Table 18. All compounds with an aromatic ring similar to the gallic acid structure have an inhibitory effect against *L. monocytogenes* **(Table 18)**.

Gallic acid is a phenolic acid that was found distributed in many plants[142] and exhibits inhibitory activity against many species of bacteria, fungus, and yeast.[143,144]

To study the effect of the MECL on bacterial growth, different concentrations (28.12 and 100 μg/mL) of MECL were tested. As compared with the control cell suspensions without carob extract, we observed a modification of the typical cell growth curves of *L. monocytogenes* **(Figure 23)**. The addition of these concentrations of our extract at cell density of 10^3 CFU/mL evidenced a longer inhibition phase (phase Lag), cell growth phase resumed by a rate lower than the control cell suspensions. In addition, cultures exposed to MECL entered in a stationary phase at a lower bacteria concentration. When the concentration of the extract was increased, from 28.12 to 100 μg/mL, the duration of the inhibition phase increased, whereas the rate of the growth after inhibition as well as the cell concentration at which stationary phase was entered decreased. The observation that the resumed growth rate is substantially lower than the uninhibited culture growth rate confirms that the inhibited cells are not totally able to metabolize compounds in the carob extract or recover from their inhibition. This phenomenon was found also on treatment of *Salmonella hadar* with aqueous garlic extract.[145]

The lower secondary growth rate could indicate either the presence of some unrepaired lesions in the cells or the depletion of limiting nutrients from the medium during the inhibition phase. The antimicrobial activity evolution of MECL during storage shows that carob extract could be stored at +4 or −18 °C because no detectable big losses of antibacterial activity at

these temperatures were noticed over 24 days **(Figure 24)**. These results suggest that the observed antimicrobial activity is probably due to the constituent polyphenols of MECL that are stable at low temperatures.

The use of the API-ZYM system does not show differences in the enzymatic profile of the bacterium. This result leads us to search and propose a mode of action of the phenolic compounds present in MECL on *L. monocytogenes*. With the use of proline, we tried to evaluate if phenolic metabolites behave as proline analogues or proline mimics. If this is the case, polyphenols can inhibit proline oxidation by inhibition of proline dehydrogenase (PDH) at the cytoplasm membrane in the prokaryotic cell and therefore inhibit the bacterium growth.[146]

In this case, the addition of proline could overcome the inhibition of proline analogues with aromatic ring structures. The antimicrobial effect of gallic acid and (−)-epigallocatechin-3-gallate was removed when 1 mM proline was added into the well. These results can give insight into the mode of action of polyphenols that might act as inhibitors of PDH and disturb the pentose phosphate pathway, confirming previous studies for other pathogenic bacteria such us *H. pylori*.[146]

Table 18: MICs of Polyphenols Found in MECL on *L. monocytogenes*[a]

Polyphenols	Technique		
	Microdilution method		Disk diffusion
	MIC (µg/mL)	+1mM proline	diameter of Inhibition zone (mm)
Isoquercitin	112.5	-	NA
gallic acid	450	++	NA
myricitrin	450	-	NA
(-)epigallocatechin-3-gallate	225	++	NA
Synergic activity			
isoquercitin+gallic acid	450	-	NA
isoquercitin+myricitrin	-	-	NA
isoquercitin+(-)epigallocatechin-3-gallate	-	-	NA
gallic acid+myricitrin	900	-	NA
gallic acid+(-)epigallocatechin-3-gallate	-	-	NA
myricitrin+(-)epigallocatechin-3-gallate	-	-	NA

NA: Not active, ++: acitve, -: not tested

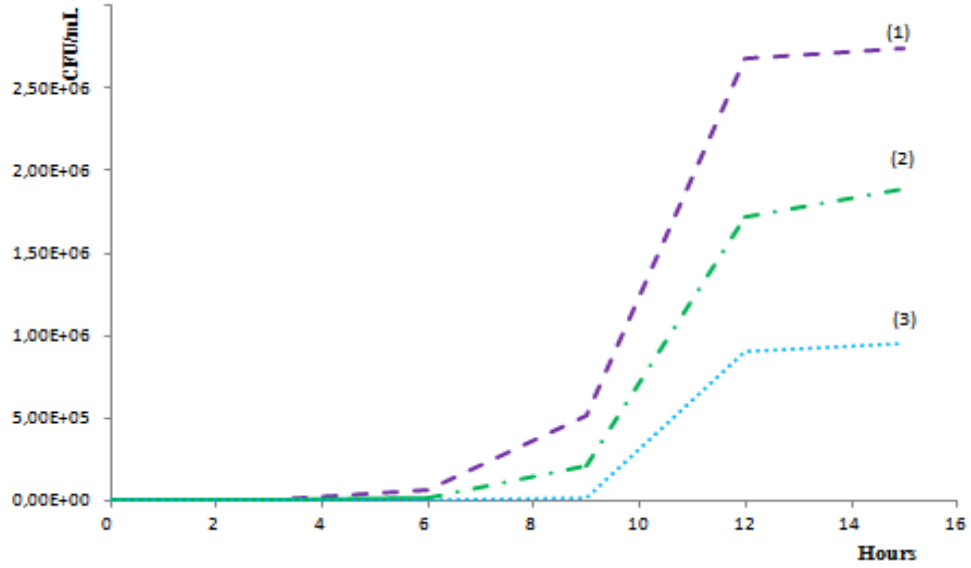


Figure 23: Changes in normal growth curve (1) of *L. monocytogenes* at 37 °C in presence of treatment of carob leaves methanol extract at different concentrations: (2) MIC = 28.12 µg/mL and (3) 100 µg/mL.

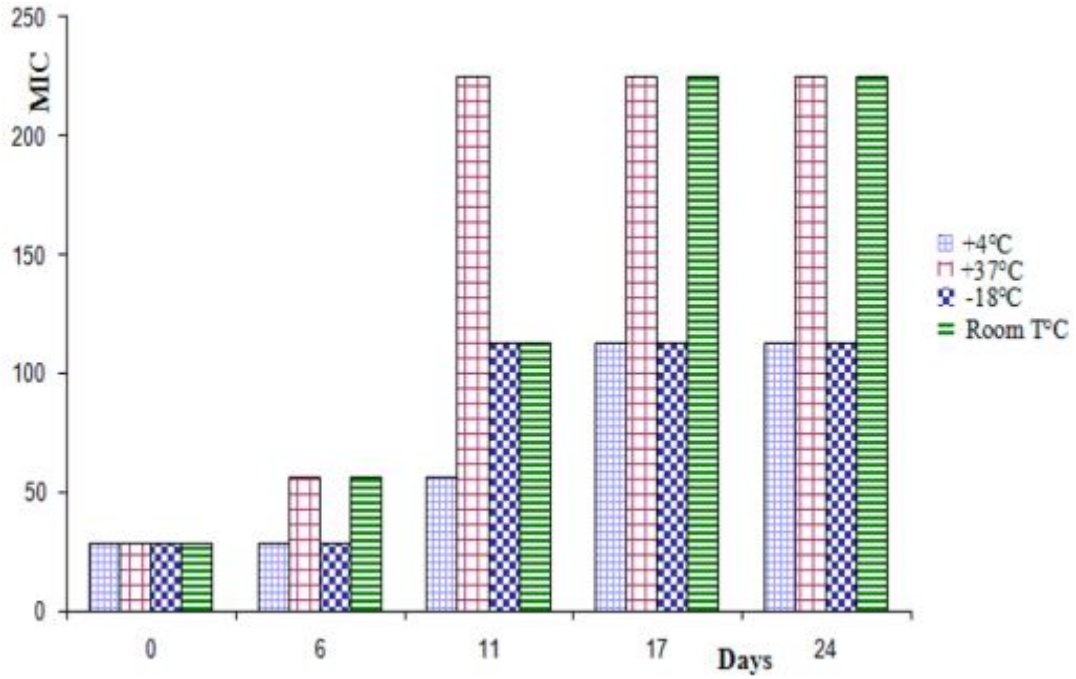


Figure 24: MIC evolution of carob leaves methanolic extract against *L. monocytogenes* during storage at different temperatures.

CHAPTER III

ACARICIDAL ACTIVITY

This work was done at the "Instituto de Ciencias Agrarias (ICA-CSIC)-Madrid, Spain" under the supervision of Prof. Azucena Gonzalez Coloma during the period April-July 2013

Acaricidal Activity of *Lavandula luisieri* Hydrolates:

I. *Lavandula luisieri*:

L. luisieri (Rozeira) **(Figure 25)** Rivas Mart. is one of the five spontaneous species of the genus Lavandula occurring in Portugal. It is endemic to the Iberian Peninsula, being very common throughout Portugal and in the Southwest of Spain.[147] *L. luisieri* is an erect bush (50 to 75 cm high) bearing light green leaves on new growth and more greyish ones at the base. Its narrow leaves grow up to the beginning of the floral head. These heads are made up of numerous small dark violet flowers, mainly vertically aligned, and by green bracts. Traditionally this species has been used as an expectorant in chest problems and colds, against headaches and migraines and for its anti-spasmodic, laxative and stimulant properties. It is also used as a disinfectant, to perfume linen and protect it against moths.[147]

During the last years, several studies have shown interesting properties of *L. luisieri* essential oils foreseeing many other applications. The biological activities so far reported for these oils include: antifeedant effects against *Leptinotarsa decemlineata*, *Myzus persicae*, *Rhopalosiphum padi*, and *Spodoptera littoralis*,[148,149] nematicidal activity against *Bursaphelenchus xylophilus*[150] and antimicrobial activities.[151]

Figure 25: *Lavandula luisieri*

II. Materials and Methods:

II. a. Chemicals:

All chemical compounds were of high-performance liquid chromatography (HPLC)-grade.

II. b. Plant material, extraction and analysis:

Plant material and cultivation

L. luisieri plants were cultivated in an experimental field (Comarca del Campo de Cariñena, Aguarón, Zaragoza, Spain).

Table 19: Altitude and average annual precipitation of the experimental field:

Altitude (m)	Average annual temperature	Average annual pecipitation
649	13º C	450 mm

Seeds collected in June 2008 from a wild population located in Pueblo Nuevo del Bullaque (Ciudad Real, Spain) (B) (latitude: 39° 27' 41" N, longitude: 4° 24' 34" W, altitude: 733 m) were multiplied in a nursery and then transferred to the field in April 2007. The aerial parts of

the cultivated plants, collected in June 2012, were dried in the absence of light at room temperature.

Extract preparation

The ethanolic extraction (EtOH) was performed in a Soxhlet apparatus and concentrated in vacuo (12.5 % yield). Hydrodistillation (EO) was performed in a Clevenger-type apparatus (0.8% yield) according to the method recommended by European Pharmacopoeia (http://www.edqm.eu/ last accessed 12/01/2014).

Water residue extraction and fractionation

The EO-free water residue was collected after the essential oil (EO) and was decanted and 155 mL extracted with dichloromethane, DCM (150 ml x 3) and NaCl to give an organic extract (230 mg, 0.15 % yield).

The organic extract was submitted to bio-guided fractionation against *Hyalomma lusitanicum* and *Rhipicephalus sanguineus* larvae. The extracts was fractioned by flash chromatography on a 2.5 cm diameter silica cartridge (40-70 µm) eluted with a DCM:MeOH gradient (100:0-95:5) and 20 ml/min flow rate in a Jones Flash Chromatography apparatus. The fractions were monitored by TLC (silica gel 60 F254, 0.25 mm, Merck) to give fractions G1 (1.1%), G2 (12.0%), G3 (4.3%) and G4 (6.6%).

Essential oil and organic fractions analysis

The essential oil (EO), its organic extract (OE-AQ) and fractions (G1-4) were analyzed by GC-MS using an Agilent 6890N gas chromatograph (Agilent Technologies, Palo Alto, California, USA) coupled to an Agilent 5973N mass detector (electron ionization, 70 eV) (Agilent Technologies, Palo Alto, California, USA) and equipped with a 25m x 0.2 mm id HP-1 (methyl polysiloxane, 0.2 µm film thickness) and a 30m x 0.25mm id Carbowax

(polyethylene glycol, 0.25 µm film thickness) capillary columns (Hewlett-Packard). Working conditions were as follows: injector temperature, 260 ºC; temperature of the transfer line connected to the mass spectrometer, 280 ºC; column temperature 70-190 ºC, 5 ºC/min. EI mass spectra and retention data were used to identify compounds by comparing them with those of standards or found in the Wiley Mass Spectral Database (2001). Quantitative data were obtained from the TIC peak areas without the use of response factors.

HPLC-MS analysis

The EtOH extract, aqueous-organic extract (OE-AQ) and its fractions (G1-4) were analyzed by HPLC-MS on a Shimadzu LC-20AD HPLC coupled to a LCMS-2020 QP mass spectrometer using an electrospray ionization (ESI) interface and a Teknokroma, Mediterranea Sea_{18} column (250 x 4.6 mm, 5 µm particle size) with an ACE 3 C18 analytical guard cartridge. The compounds were eluted with methanol (MeOH): 0.1% acetic acid in milli-Q water 38:100% gradient for 45 min, 100% MeOH for 10 min and 100:38% for 13 min at 0.5 mL/min and 15 L/min nitrogen (drying gas for solvent evaporation) flow rates. The electrospray capillary potential was set to +4.50kV and ESI was conducted in the Full Scan positive mode (m/z = 145-545) with a potential of 1.30 kV and a capillary temperature of 250º C. Stock solutions of extracts (0.25 µg/µL), compounds 1, 5, 8 (isolated from *L. luisieri*) and oleanolic acid (Sigma) (0.05 µg/µL) were dissolved in MeOH for sample injection (10 µL). All the solvents used were HPLC-MS grade.

II. c. Acaricidal assay:

Spanish populations of *Hyalomma lusitanicum* and *Rhipicephalus sanguineus* from Central Spain (Ciudad Real and Madrid) were used. Engorged female ticks were collected on hosts (*H. lusitanicum* on deer and *R. sanguineus* on dog) and maintained at 22-24 ºC and 70% RH until oviposition. The eggs were kept under the same environmental conditions until they

hatched. 4-6 Weeks old larvae **(Figure 26)** were used for the bioassays carried out as described.[152] Briefly, 25 mg of cellulose were mixed with 50 µl of the organic extract at different concentrations and the solvent allowed to evaporate. Acibelte (cypermethrin 0,5%) was used as positive control along with a blank test (cellulose) and a negative control (solvent plus cellulose). For each test, three replicates with 20 larvae each were used. Paralyzed and dead ticks were counted after 24h of incubation with the treated cellulose at the environmental conditions described, using a binocular magnifying glass.

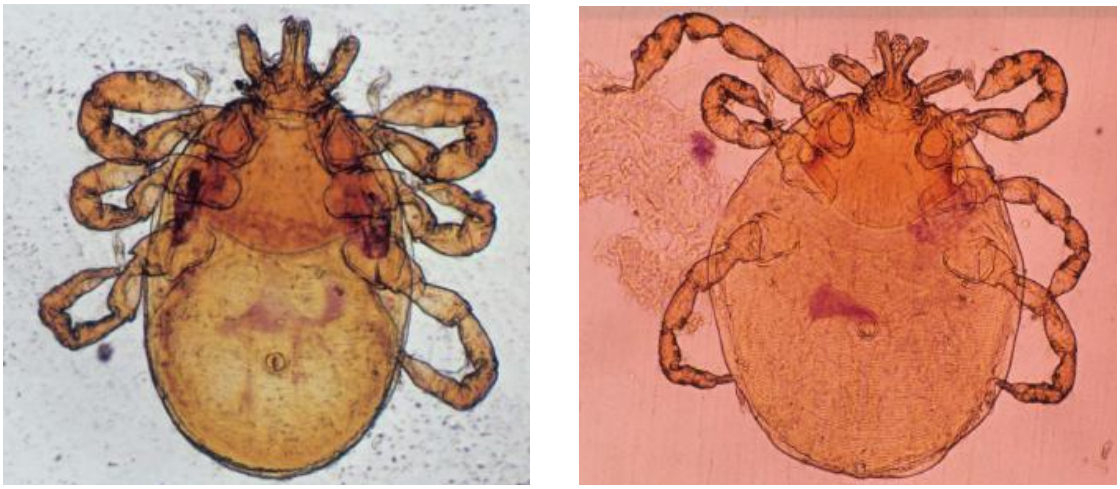


Figure 26: Larval stage of *Hyalomma lusitanicum* (right) and *Rhipicephalus sanguineus* (left)

III. Results:

Table 20 shows the ixodicidal effects of the essential oil (EO), organic extracts (ETOH and OE-AQ) and fractions G2-4 from the OE-AQ. Both the EO and the OE-AQ extracts were very active against both tick species tested, with the OE-AQ extract being more effective on *H. lusitanicum* at lower doses. The bioassay of the OE-AQ fractions indicated that the active components against both tick species were concentrated in fraction G2. Fractions G3 and G4 were more active on *R. sanguineus*.

The chemical analysis showed that the major component of the EO was camphor. The OE-AQ was mainly composed of camphor, ,3,4,4-tetramethyl-5-methylidenecyclopent-2-en-1-one , 5-hydroxymethyl-2,3,4,4-tetramethylcyclopent-2-en-1-one and (2,2,3,4-tetramethyl-5-oxocyclopent-3-en-1-yl)-methyl acetate, G2 contained 5-hydroxymethyl-2,3,4,4-tetramethylcyclopent-2-en-1-one and (2,2,3,4-tetramethyl-5-oxocyclopent-3-en-1-yl)-methyl acetate and the inactive EtOH extract had oxo-cadinol and tormentic acid as the major components **(Table 21)**: None of these compounds has been described as having acaricidal effects.

Table 20: Activity of organic extracts and fractions of *L. luisieri* against *H. lusitanicum* and *R. sanguineus*.

Extract	**Concentration**	**Paralyzed (%)***	
	(mg/mg cellulose)	***H. lusitanicum***	***R. sangineus***
EO	40	100±0.0	95.0±0.3
	20	8.8±0.6	NT
EtOH	40	10.5±2.4	26.4±0.1
OE-AQ	40	100±0.0	100±0.4
	20	100±0.0	91.9±0.4
	10	100±0.0	48.3±5.7
	5	38.8±1.1	NT
G2	40	100±0.0	96.6±0.1
	20	100±0.0	100±0.0
	10	94.2±0.1	93.3±1.6
	5	71.7±0.3	NT
G3	40	66.3±6.2	95.2±0.1
G4	40	9.9±1.1	77.7±0.3

*Paralisis $\geq$ movement in one leg after stimulation.
NT, not tested

Table 21: Chemical composition of the different *L. Luisieri* extracts.

Compd	% GC			% LC
	EO	**OE-AQ**	**G2**	**EtOH**
Camphene	1.57	-	-	-
1,8-cineole	1.95	-	-	-
Fenchone	2.85	-	-	-
Camphor	60.33	49.4	-	-
2,3,4,4-tetramethyl-5-methylidenecyclopent-2-en-1-one	8.48	19.7	-	-
D-Verbenone	1.17	2.5	-	-
Exobornyl acetate	4.64	-	-	-
Cis-α-Necrodyl acetate	1.90	-	-	-
5-Hydroxymethyl-2,3,4,4-tetramethylcyclopent-2-en-1-one	-	10.1	20.05	4.99
(2,2,3,4-tetramethyl-5-oxocyclopent-3-en-1-yl)-methyl acetate	0.66	7.7	29.08	9.71
3-Oxo-cadinol	-	-	-	16.87
Tormentic acid	-	-	-	14.95
Tormentic acid isomer	-	-	-	3.16
Ursolic acid	-	-	-	4.48

Much of the scientific research on lavandula essential oil activity and composition were reported, but to our knowledge and literary survey, there is no report available on the chemical characterisation of hydrolates and polar compounds of this plant except the work of Libran et al. who found 17 and 26 compounds in *L. angustifolia* Mill. and *L. hybrida* aqueous extract respectively composed by linear monoterpenes, cyclic monoterpenes, aromatic monoterpenes and other compounds.[153]

There are multiple reports on ixodicidal toxic and repellent effects of plant-derived essential oils.[154] However this is the first report on *L. luisieri* EO and hydrolate fractions containing necrodane-type monoterpenes. *L. angustifolia* EO showed strong acaricidal effects against R. (B.) *annulatus* showing significant acaricidal activity even at 6 hpi after 1 min exposure of adult female ticks to 8.0% of essential oil.[155]

FINAL DISCUSSION

Plants produce thousands of organic compounds that are traditionally divided into two large classes primary and secondary metabolites. Primary metabolites are essential for plant growth and development and the majority appears to be common in all plants. Secondary metabolites often act as defence molecules and protect plants in various adverse conditions and were once thought to be non essential for plant growth and development. Plant secondary metabolites are diverse in chemical nature. Biosynthesis of secondary metabolites starts from basic pathways, such as the glycolysis or shikimic acid pathways, and subsequently diversifies, largely depending on cell type, developmental stage and environmental cues. Based on chemical composition, secondary metabolites are broadly divided into two groups: nitrogen-containing molecules (alkaloids) and nitrogen-deficient molecules (terpenoids and phenolics).[157]

Nowadays, allelopathy of secondary metabolites is being explored and research is ongoing in field crop production for integrated pest and desease management. Allelochemistry, the production and release of toxic chemicals produced by one species that affect a receiving susceptible species, has been the subject of diverse groups of scientific community. Allelopathy defined as chemically elicited interactions between plants or pathogens is mediated by secondary metabolites type of compounds.[158] 'Novel weapons hypothesis' was first proposed by Callaway and Aschehoug for plant species stated that « Exotic plants might release allelochemical compounds that are novel species in the new range contributing to its invasive success.»[159] Allelochimicals are bioactive compounds which play a vital role as natural pesticides and can resolve problems like pest biotypes, health defects, soil and environment pollution resulting in climate change caused by the indiscriminate use of synthetic agrochemicals.[160]

In this thesis we dimonstrate that α, β, γ, δ unsaturated C10 and heteroaromatic aldehydes as secondary metabolites are generally more potent nematicidal than their shortest C chain counter parts versus *Meilodogyne* spp. in vitro experiments due to the presence of the

carbonyl carbon atom, an electrophilic site that may easily react with primary amino and thiol groups, resulting in formation of Schiff bases and hemithioacetals, respectively with amine and thiol groups of V-ATPase. We suppose that this is the same mode of action also for isothiocyanates due to same mode of death of nematodes in straight shape.

Studies to more understand the mode of action of these molecules as nematicides are in progress now using cell cultures.

We showed also that polyphenols are reponsible of the high antilisterial activity of methanol extract from carob leaves and this may be by inhibiting the oxidation of proline by proline dehydrogenase due to mimic structure of polyphenols to proline. In the future, we will study the antilisterial activity of carob leaves methanol extract *in vivo* using real substrates such as meat, cheese and fish samples.

The dichloromethane exctract from *Lavandula luisieri* hydrolates showes an acaricidal activity against *Hyalomma lusitanicum* and *Rhipicephalus sanguineus* juvinile stage ticks. This result must be more investigated in order to fix the EC50 and to identify the active molecules and maybe using animals models like dogs and rabbits.

REFERENCES

(1) Verpoorte, R., Pharmacognosy in the New Millenium: Leadfinding and Biotechnology. *J Pharm Pharmacol,* **2000**, 52: 253-262.

(2) Gurib-Fakim, A., Medicinal plants: Trad itions of yesterday and drugs of tomorrow. *Mol Aspects Med,* **2006**, 27: 1-93.

(3) Von Maydell, H. J., Trees and Shrubs of the Sahel. Verlag Josef Margraf, Weikersheim, p. 562.

(4) Hedberg, I., Hedberg, O., 1982. Inventory of plants used in tradi-tional medicine in Tanzania. I. Plants of the families Acanthaceae-Cucurbitaceae. *J Ethnopharmocol,* **1982**, 6, 29-60.

(5) Farnsworth, N. R., Screening plants for new medicines. In: Wilson, E.O. (Ed.), *Biodiversity*. National Academic Press, Washington, DC, **1988**, pp. 83-97.

(6) Sheldon, J., Balick, M. J., Laird, S. A., 1997. Medicinal Plants: Can Utilization and Conservation Coexist? The New York Botanical Garden, Bronx, New York, USA, **1997**.

(7) Kossel, A., Uber die Chemische Zusammensetzung der Zelle, Archiv fur Physiologie **1891**, 181-186.

(8) Czapek, F., Spezielle Biochemie, Biochemie der Pflanzen, G. Fischer Jena, **1921**, 3. 369.

(9) Harborne, J. B., Classes and functions of secondary products, in: N.J. Walton, D.E. Brown (Eds.), Chemicals from Plants, Perspectives on Secondary Plant Products, Imperial College Press, **1999**, pp. 1-25.

(10) Bourgaud, F., Gravot, A., Milesi, S., Gontier, E. Production of plant secondary metabolites: a historical perspective, review. *Plant Sci,* **2001**,*161*, 839-851.

(11) Chitwood, D. J. Phytochemical based strategies for nematode control (Review). *Annu. Rev. Phytopathol*. **2002**, *40*, 221-249.

(12) Li, J. Ou-Lee, T. M., Raba, R., Amundson, R. G., Last, R. L., Arabidopsis Flavonoid mutants are hypersensitive to UV-B radiation, *Plant Cell*, **1993**, 5, 171-179.

(13) Rice, E. L. *Allelopathy*; Academic Press: New York, **1984**.

(14) Isman, M. B. Botanical insecticides, deterrents, and repellents in modern agriculture and an increasingly regulated world. *Annu. Rev. Entomol*. **2006**, *51* ,45-66.

(15) Copp, B. R., Antimicobacterial natural products. *Nat Prod* Rep, **2003**, *20*, 535-557.

(16) Gaugler, R. & Bilgrami, A. L. *Nematode behaviour*. Wallingford, UK: CABI Publishing. **2004**

(17) Jones, J. T., Haegeman, A., Danchin, E, G, J., Gaur, H, S., Heilder, J., Jones, G, K., Kikuchi, T., Manzanilla-Lopez, R., Palomaers-Rius, J. E., Wim, M. L., Esmael, W, M, L., Perry, R, N. Top 10 plant-parasitic nematodes in molecular plant pathology. *Mol. Plant Pathol (review)* **2013**, *14*(9), 946-961.

(18) 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.

(19) Sumner, D. R., and A. W. Johnson. Effect of root-knot nematodes on Fusarium wilt of watermelon. *Phytopathology*, **1973**, *63*, 857-861.

(20) Sasanelli, N. Tables of nematode-pathogenicity. *Nematol. Mediterr*. **1994**, *22*, 153-157.

(21) Ntalli, G. N.; Caboni, P. Botanical Nematicides: A Review. *J. Agric. Food Chem*. **2012**, *60*, 9929-9940.

(22) Bird, A. F. The attractiveness of roots to the plant-parasitic nematodes *Meloidogyne javanica* and *M. hapla*. *Nematologica*, **1959**, *4*, 322–335.

(23) Young, I. M., Griffiths, B. S. & Robertson, W. M. Continuous foraging by bacterial-feeding nematodes. *Nematologica*, **1996**, *42*, 378–382.

(24) Davis, R. F., Effect of *Meloidogyne incognita* on watermelon yield. *Nematropica*, **2007**, 37:287-293.

(25) Caboni, P., Ntalli, G. N., 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.

(26) Narsaiah, K., Narayan Jha, S. N., Bhardwaj, R., Sharma, R., Kumar, R. Optical biosensors for food quality and safety assurance. *J. Food Sci Technol*. **2012**, *49* (4), 383-406.

(27) Petit, L., Gibert, M., Popoff, M. R. *Clostridium perfringens*: toxinotype and genotype. *Trends Microbiol*. **1999**, 7, 104-110.

(28) Settanni, L.; Corsetti, A. The use of multiplex PCR to detect and differentiate food and beverage-associated microorganisms: A review.*J. Microbiol. Meth*. **2007**, *69*, 1–22.

(29) Doan, C. H., Davidson, P. M., 1999. Growth and production of enterotoxin A by *Staphylococcus aureus* on home style French fries. *J. Food Sci*. **1999**, *64*, 913–917.

(30) Palmer, A. C., Kishony, R. Understanding, predicting and manipulating the genotypic evolution of antibiotic resistance. *Nat. Rev. Genet*. **2013**, *14*, 243-248.

(31) Meyer, E., Schwab, F., Schroeren-Boersch, B.; Gastmeier, P. Dramatic increase of third-generation cephalosporin-resistant *E. coli* in German intensive care units: Secular trends in antibiotic drug use and bacterial resistance, 2001 to 2008. *Crit. Care*, **2010**, *14*:R113.

(32) Spellberg, B., Guidos, R., Gilbert, D., Bradley, J., Boucher, H. W., Scheld, W. M., Bartlett, J. G., Edwards, J., J. R. The epidemic of antibiotic-resistant infections: A call to action for the medical community from the infectious diseases society of America. *Clin. Infect. Dis.* **2008**, *46*, 155-164.

(33) Aissani, N., Coroneo, V., Fattouch, S., Caboni, P. Inhibitory Effect of Carob (*Ceratonia siliqua*) Leaves Methanolic Extract on *Listeria monocytogenes*. *J. Agric. Food Chem*. **2012**, *60*, 9954-9958.

(34) US Food and Drug Administration. 2012. Final Update on Multistate Outbreak of Listeriosis Linked to Whole Cantaloupes. http://www.fda.gov/food/foodsafety/corenetwork/ucm272372.htm.

(35) US Food and Drug Administration. 2011-2013, posting date. All Recalls. FDA. http://www.fda.gov/Safety/Recalls/default.htm.

(36) Habeck, M. Vaccinating against ticks. *DDT*, **2002**, *7* (11) 586-588.

(37) d'Oliveira C. *Theileria annulata*: recombinant vaccine development and molecular identification. PhD thesis. University of Utrecht; 1997:120.

(38) Willadsen, P. Tick control: thoughts on a research agenda. *Vet. Parasitol*. **2006**, *138*, 161-168.

(39) Assenga, S. P., M. You, C. H. Shy, J. Yamagishi, T. Sakaguchi, J. Zhou, M. K. Kibe, X. Xuan, and K. Fujisaki. The use of a recombinant baculovirus expressing a chitinase from

the hard tick *Haemaphysalis longicornis* and its potential application as a bioacaricide for tick control. *Parasitol. Res*. **2006**, 98: 111-118.

(40) Maranga, R. O., Kaaya, G. P., Mueke, J. M., and Hassanali, A. Effects of combining the fungi *Beauveria bassiana* and *Metarhizium anisopliae* on the mortality of the tick *Amblyomma variegatum* (ixodidae) in relation to seasonal changes. *Mycopathologia*, **2005**, *159*, 527-532.

(41) Hassan, S. M., Dipeolu, O. O., Amoo, A. O., and Odhi-ambo, T. R. Predation on livestock ticks by chickens. *Vet. Parasitol*. **1991**, *38*, 199-204.

(42) Gazim, Z. C., Demarchi, I. G., Lonardoni, M. V., Amorim, A. C., Hovell, A. M., Rezende, C. M., Ferreira, G. A., Lima, E. L., Cosmo, F. A., Cortez, D. A. Acaricidal activity of the essential oil from *Tetradenia riparia* (Lamiaceae) on the cattle tick *Rhipicephalus* (Boophilus) *microplus* (Acari; Ixodidae). *Exper. Parasitol*. **2011,** *384* (129), 175–178.

(43) Zorloni, A., Penzhorn, B. L., Eloff, J. N. Extracts of *Calpurnia aurea* leaves from southern Ethiopia attract and immobilise or kill ticks. *Vet. Parasitol.* **2010**, *168*, 160-164.

(44) Heisey, R. M. Allelopathy and the secret life of *Ailanthus altissima*. *Allelopathy*. **1997**, 28-36.

(45) De Martino, L., De Feo, V. Chemistry and biological activities of *Ailanthus altissima* swingle: A review. *Pharmacogn. Rev*. **2008**, *2*, 339-350.

(46) Kraus, W., Koll-Weber, M.; Maile, R., Wunder, T., Vogler, B. Biologically active constituents of tropical and subtropical plants. *Pure Appl. Chem*. **1994**, *66*, 2347−2352.

(47) Puntener, W. *Manual for Field Trials in Plant Protection*, 2nd ed.; Ciba Geigy: Basel, Switzerland, **1981**, 205.

(48) Seefeldt, S. S., Jensen, J. E., Fuerst, E. P. Log-logistic analysis of herbicide rate response relationships. *Weed Technol*. **1995**, 9, 218-227.

(49) Kim, J., Seo, S. M., Lee, S. G., Shin, S. C., Park, I. K. Nematicidal activity of plant essential oils and components from coriander (*Coriandrum sativum*), oriental sweet gum (*Liquidambar orientalis*), and valerian (*Valeriana wallichii*), essential oils against pie wood nematode (*Bursaphelenchus xylophilus*). *J. Agric. Food Chem*. **2008**, *56*, 7316-7320.

(50) Andersen, R. A.; Hamilton-Kemp, T. R., Hildebrand, D. F., Mc Cracken, C. T., Collins, R. W., Fleming, P. D. Structure-antifungal activity relationship among volatile C6 and C9 aliphatic aldehydes, ketones, and alcohols. *J. Agric. Food Chem.* **1994**, *42*, 1563-1568.

(51) Kong, J. O., Lee, S. M., Moon, Y. S., Lee, S. G., Ahn, Y. J. Nematicidal activity of plant essential oils against *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae). *J. Asia-Pac. Entomol*. **2006**, *9*, 173-178.

(52) 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.

(53) Luo, H., Mo, M. H., Huang, X. W., Li, X., Zhang, K. Q. *Coprinus comatus*: A basidiomycete fungus forms novel spiny structures and infects nematodes. *Mycologia* **2004**, *96*, 1218-1225.

(54) Luo, H., Liu, Y., Fang, L., Li, X., Tang, N., Zhang, K. *Coprinus comatus* damages nematode cuticle mechanically with spiny ball and produces potent toxin to immobilize nematodes. *Appl. Environ. Microbiol*. **2007**, 3916-3923.

(55) Luo, H., Li, X., Li, G., Pan, Y., Zhang, K. Acanthocytes of *Stropharia rugosoannulata* function as a nematode-attacking device. *Appl. Environ. Microbiol*. **2006**, 2982-2987.

(56) Ansar, M., Al Akoum Ebriki, S., Mouhoub, R., Berthelot, P., Vaccher, C., Vaccher, M. P., Flouquet, N., Caignard, D. H., Renard, P., Pirard, B., Rettori, M. C., Evrard, G., Durant, F., Debaert, M. 3-Benzo[b]furyl- and 3-benzo[b]thienylaminobutyric acids as GABAB ligands: synthesis and structure-activity relationship studies. *Eur. J. Med. Chem.***1996**, *31*, 449-460.

(57) Annunziata, R., Benaglia, M., Cinquini, M., Cozzi, F., Tocco, G. *Org. Lett*. **2000**, *2*, 1737-1739.

(58) Zhang, Z., Marshall, A. G. A universal algorithm for fast and automated charge state deconvolution of electrospray mass-to-charge ratio spectra. *J. Am. Soc. Mass Spectrom*. **1998**, *9*, 225-233.

(59) Ong, S. E., Mann, M. Mass spectrometry-based proteomics turns quantitative. *Nat. Chem. Biol*. **2005**, 252-262.

(60) 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**, *14*, 7856-7863.

(61) Davies, K. G.; Curtis, R. H. C. Cuticle surface coat of plant-parasitic nematodes. *Annu. Rev. Phytopathol*. **2011**, *49*, 135-156.

(62) Eisenmann, D. M. *Wnt Signaling*; The *C. elegans* Research Community, WormBook, 2005; http://www.wormbook.org.

(63) Johnstone, I. L. Cuticle collagengenes expression in *Caenorhabditis elegans*. *Trends Genet*. **2000**, *16*,21-27.

(64) Ahlfors, S. R., Sterner, O., Hansson, C. Reactivity of contact allergenic haptens to amino acid residues in a model carrier peptide, and characterization of formed peptide-hapten adducts. *Skin Pharmacol. Appl. Skin Physiol*. **2003**, *16*, 59-68.

(65) Nilsson, A. M., Bergstrom, M. A., Luthman, K., Nilsson, J. L., Karlberg, A. T. An *α,β*-unsaturated oxime identified as a strong contact allergen. Indications of antigen formation via several pathways. *Food Chem. Toxicol*. **2005**, *43*, 1627-1636.

(66) Natsch, A., Gfeller, H. LC-MS-based characterization of the peptide reactivity of chemicals to improve the in vitro prediction of the skin sensitization potential. *Toxicol. Sci*. **2008**, *106*, 464-478.

(67) Koltai, H., Chejanovsky, N., Raccah, B., Spiegel, Y. The first isolated collagen gene of the root-knot nematode *Meloidogyne javanica* is developmentally regulated. *Gene* **1997**, *196*, 191-199.

(68) Roberts, D. W., Natsch, A. High throughput kinetic profiling approach for covalent binding to peptides: application to skin sensitization potency of Michael acceptor electrophiles. *Chem. Res. Toxicol*. **2009**, *22*, 592-603.

(69) Klopman, G. Chemical reactivity and the concept of charge-and frontier-controlled reactions. *J. Am. Chem. Soc*. **1968**, *90*, 223-234.

(70) Roy, R. K., Bagaria, P., Naik, S., Kavala, V., Patel, B. K. Chemoselectivities in acetalization, thioacetalization, oxathioacetaliza-tion and azathioacetalization. *J. Phys. Chem. A.* **2006**, *110*, 2181-2187.

(71) Wymore, T., Deerfield, D. W., Field, M. J., Hempel, J., Nicholas, H. B., J. R. Initial catalytic events in class 3 aldehyde dehydrogenase: MM and QM/MM simulations. *Chem.−Biol. Interact*. **2003**, *143−144*, 75-84.

(72) Bonnefont-Rousselot, D. Glucose and reactive oxygen species. *Curr. Opin. Clin. Nutr. Metab. Care*, **2002**, *5*, 561-568.

(73) Arinbasarova, A. Y.; Medentsev, A. G.; Krupyanko, V. I. Gossypol inhibits electron transport and stimulates ROS generation in Yarrowia lipolytica mitochondria. *Open Biochem J.* **2012**, *6*, 11-15.

(74) Gluck, S. V-ATPases of the plasma membrane. *J. Exp. Biol*. **1992**, *172*, 29-37.

(75) Breton, S., Smith, P. J., Lui, B., Brown, D. Acidification of the male reproductive tract by a proton pumping (H+)-ATPase. *Nat. Med*. **1996**, *2*, 470-472.

(76) Lee, S. K., Li, W., Ryu, S. E.; Rhim, T., Ahnn, J. Vacuolar (H+)-ATPases in *Caenorhabditis elegans*: what can we learn about giant H+ pumps from tiny worms? *Biochim. Biophys. Acta,* **2010**, *1797*, 1687-1695.

(77) Knight, A. J., Behm, C. A. Minireview: The role of the vacuolar ATPase in nematodes. *Exp. Parasitol*. **2012**, *132*, 47−55.

(78) Hager, A., Lanz, C. Essential sulfhydryl groups in the catalytic center of the tonoplast H+-ATPase from coleoptiles of *Zea mays* L. as demonstrated by the biotin-streptavidin-peroxidase system. *Planta*, **1989**, *180*, 116-122.

(79) Seidel, T., Scholl, S., Krebs, M.; Rienmu¨ller, F., Marten, I., Hedrich, R., Hanitzsch, M., Janetzki, P., Dietz, K. J. Schumacher, K. Regulation of the V-Type ATPase by redox-modulation. *Biochem. J*. **2012**, *448*, 243-251.

(80) Okahashi, N., Nakamura, I., Jimi, E., Koide, M., Suda, T., Nishihara, T. Specific inhibitors of vacuolar H(+)-ATPase trigger apoptotic cell death of osteoclasts. *J. Bone Miner. Res.* **1997**, *12*, 1116-1123.

(81) Caboni, P., Sarais, G., Aissani, N., Tocco, G., Sasanelli, N., Liori, B., Carta, A., Angioni, A. Nematicidal activity of 2-thiophenecarbox-aldehyde and methylisothiocyanate from caper (*Capparis spinosa*) against *Meloidogyne incognita*. *J. Agric. Food Chem*. **2012**, *60*, 7345-7351.

(82) Liégeois, S., Benedetto, A., Michaux, G., Belliard, G., Labouesse, M. Genes required for osmoregulation and apical secret ion in *Caenorhabditis elegans*. *Genetics,* **2007**, *175*, 709-724.

(83) Nelson, F. K., Riddle, D. L. Functional study of the *Caenorhabditis elegans* secretory-excretory system using laser micro-surgery. *J. Exp. Zool*. **1984**, *231*, 45-56.

(84) Mahajan-Miklos, S., Tan, M. W., Rahme, L. G., Ausubel, F. M. Molecular mechanisms of bacterial virulence elucidated using a *Pseudomonas aeruginosa−Caenorhabditis elegans* pathogenesis model. *Cell*, **1999**, *96*, 47-56.

(85) Kim, J. H., Chan, K. L., Mahoney, N., Campbell, B. C. Antifungal activity of redox-active benzaldehydes that target cellular antioxidation. *Ann. Clin. Microbiol. Antimicrob.* **2011**, 23.

(86) Tesoriere, L., Butera, D., Gentile, C., Livrea, M. A. Bioactive components of caper (*Cappari s spinosa* L.) from Sicily and anti-oxidant effect s in a red meat simulated gastric digestion. *J. Agric. Food Chem*. **2007**, *55*, 8465-8471.

(87) Zhou, H, Jian, R, Kang, J, Huang, X, Li, Y, Zhuang, C, Yang, F, Zhang, L, Fan, X, Wu, T, Wu, X. Anti-inflammatory effects of caper (*Capparis spinosa* L.) fruit aqueous extract and the isolation of main phytochemicals. *J. Agric. Food Chem*. **2010**, *58* (24), 12717-12721.

(88) Brevard. H., Brambilla, M., Chaintreau, A., Marion, J. P.Occurrence of elemental suphur in capers (*Capparis spinosa* L) and first investigation of the flavor profile. *Flavour Fragrance J*. **1992**, *7*, 313-321.

(89) Sharaf, M., El-Ansari, M. A., Saleh, N. A. M. Flavonoids of four cleome and three *Capparis species*. *Biochem Syst Ecol.* **1997**, *25*, 161-166.

(90) Harborne, J. B., Williams, C. A. Advances in flavonoid research since 1992. *Phytochem.* **2000**, *55*, 481-504.

(91) Grainge, M., Ahmed, S. *Handbook of Plants with Pest-Control Properties*; J. Wiley and Sons: New York, **1988**, pp 238-248.

(92) Brown, P. D., Morra, M. J. Control of soil-borne plant pests using glucosinolate-containing plants. *Adv. Agron*. **1997**, *61*, 167-231.

(93) Seck, D., Lognay, G., Haubruge, E., Whatelet, J. P., Marlier, M., Gaspar, C., Severin, M. Biological activity of the shrub Boscia senegalensis (Pers.) Lam. ex Poir. (*Capparaceae*) on stored grain insects. *J. Chem. Ecol*. **1993**, *19*, 377-389.

(94) Serra, B., Rosa, E., Iori, R., Barillari, J., Cardoso, A., Abreu, C., Rollin, P. *In vitro* activity of 2-phenylethyl glucosinolate, and its hydrolys is derivatives on the root-knot nematode *Globodera rostoc*hiensis. *Sci. Horticult*. **2002**, *92*,75-81.

(95) Soler-Serratosa, N., Kokalis-Burelle, N., Rodriguez-Kabana, R., Weaver, C. F., King, P. S. Allelochemicals for control of plant-parasitic nematodes. 1. In vivo nematicidal efficacy of thymol and thymol/benzaldeyde combinations. *Nematropica,* **1996**, *26*, 57-71.

(96) Luo, H., Mo, M. H., Huang, X. W., Li, X., Zhang, K. Q. *Coprinus comatus*: A basidiomycete fungus forms novel spiny structures and infects nematodes. *Mycologia* **2004**, 96.

(97) Luo, H., Liu, Y., Fang, L., Li, X., Tang, N., Zhang, K. *Coprinus comatus* damages nematode cuticle mechanically with spiny ball and produces potent toxins to immobilize nematodes. *Appl. Environ. Microbiol*. **2008**, 3916-3923.

(98) Niu, X. M., Wang, Y. L., Chu, Y. S., Xue, H. X., Li, N; Wei, L. X., M o, M. H., Zhang, K . Q. Nematodetoxic aurovertin-type metabolites from a root-knot nematode parasitic fungus *Pochonia chlamydosporia*. *J. Agric. Food Chem*. **2010**, *58* (2), 828-834.

(99) Ebel, R., Lardy, H. Influence of aurovertin on mitochondrial ATPase activity. *J. Biol. Chem*. **1975**, *250*, 4992-4995.

(100) Hudocecova, A., Hasplova, K., Kellowska, L., Ikreniova, I., Miadokova, E., Galova, E., Horvathova, E., Vaculcikova, D. Gentiana as clepiadea and Armoracia rusticana can modulate the adaptive response induced by zeoc in in human lymphocytes. *Neoplasma.* **2012**, *59* (1), 62-69.

(101) Agneta, R., Rivelli, A. R., Ventrella, E., Lelario, F., Sarli, G., Bufo, S. A. Investigation of glucosinolate profile and quantitative aspects in sprouts and roots of horseradish (*Armoracia rusticana*) using LC-ESI-Hybrid linear ion trap with Fourier transform ion cyclotron resonance mass spectrometry and infrared multiphoton dissociation. *J. Agric. Food Chem*. **2012**, *60*, 7474-7478.

(102) Weil, M. J., Zhang, Y., Nair, M. G. Tumor cell proliferation and ciclooxygenase inhibitory constituents in horse radish (*Armoracia rusticana*) and wasabi (*Wasabia japonica*). *J. Agric. Food Chem.* **2005**, *53*, 1440-1444.

(103) Tedeschi, P., Leis, M., Pezzi, M., Civolani, S., Maietti, A., Brandolini, V. Insecticidal activity and fungitoxicity of plant extracts and components of horseradish (*Armoracia rusticana*) and garlic (*Allium sativum*). *J. Environ. Sci. Health, Part B.* **2011**, *46*, 486-490.

(104) Bones, A. M., Rossiter, J. T. The enzymic and chemically induced decomposition of glucosinolates. *Phytochemistry.* **2006**, *67*, 1053-1067.

(105) Zasada, I. A., Ferris, H. Sensitivity of *Meloidogyne javanica* and Tylenchulus semipenetrans to isothiocyanates in laboratory assays. *Nematology,* **2003**, *93*, 747-750.

(106) Fleming, C. C., Turner, S. J., Hunt, M. Management of root knot nematodes in turfgra ssus ing m ustard formulations and biostimulants. *Commun. Agric. Appl. Biol. Sci.* **2006**, *71*, 653-658.

(107) Lazzeri, L., Tacconi, R.; Palmieri, S. In vitro activity of some glucosinolates and their reaction products toward a population of the nematode Heterodera schachtii. *J. Agric. Food Chem.* **1993**, *41*, 825-829.

(108) Nakamura, Y., Miyoshi, M. Electrophiles in food; the current status of isothiocyanates and their chemical biology. *Biosci. Biotechnol. Biochem.* **2010**, *74*, 242-255.

(109) Mi, L., Xiao, Z., Hood, B. L., Dakshanamurthy, S., Wang, X., Govind, S., Conrads, T. P., Veenstra, T. D., Chung, F. L. Covalent binding to tubulin by isothiocyanates: a mechanism of cell growth arrest and apoptosis. *J. Biol. Chem.* **2008**, *283*, 22136-22146.

(110) Aoudia, H., Ntalli, N., Aissani, N., Yahiaoui-Zaidi, R., Caboni, P. Nematotoxic phenolic compounds from *Melia azedarach* against *Meloidogyne incognita*. *J. Agric. Food Chem.* **2012**, *28* (60), 11675-11680.

(111) Borek, V., Morra, M. J., Brown, P. D., McCaffrey, J. P. Transformation of the glucosi nolate-derived allelochemicals allylisothiocyanate and allylnitrile in soil. *J. Agric. Food Chem.* **1995**, *43*, 1935-1940.

(112) Gimsing, A. L., Strobel, B. W., Hansen, H. C. B. Degradation and sorption of 2-propenyl and benzy lisothiocyanate in soil. Environ. *Toxicol. Chem*. **2009**, *6*, 1178-1184.

(113) Klose, S., Acosta-Martinez,V., Ajwa, H. A. Microbial community composition and enzyme activities in a sandy loam soil after fumigation with methyl bromide or alternative biocides. *Soil Biol*. Biochem. **2006**, *38*, 1243-1254.

(114) Mabberley, D. J. "A Monograph of *Melia* in Asia and the Pacific: The history of White Cedar and Persian Lilac". *The Gardens' Bulletin Singapore.* **1984**, *37* (1): 49-64.

(115) Floyd, A. G., Rainforest Trees of Mainland South-eastern Australia, *Inkata Press* **1989**, ISBN 0-909605-57-2.

(116) Khan. A. B., Ahmed, Q. U., Mir. M. R., Shukla. I., Khan. A. A. Antibacterial efficacy of the seed extracts of *Melia azedarach* against some hospital isolated human pathogenic bacterial strains. *Asian Pac J Trop Biomed.* **2011**, *1*(6): 452-455.

(117) Akhtar, Y., Young, Y. R., Isman, M. B. Comparative bioactivity of selected extracts from Meliaceae and some commercial botanical insecticides against two noctuid caterpillars, *Trichoplusia ni* and *Pseudoletia unipunctat*. *Phytochem Rev* **2008**, *7*, 77-88.

(118) Charleston, D. S, Kfir, R., Vet, L. E., Dicke, M. Behavior responses of diamond back moth *Plutella xylostella* (Lepidoptera: Plutellidae) to extracts derived from *Melia azedarach* and *Azadirachta indica*. *Bull Entomol Res,* **2005**, *95*, 457-465.

(119) Nahak, G., Sahu, R. K. In vitro antioxidative activity of *Azadirachta indica* and *Melia azedarach* Leaves by DPPH scavenging assay. *J. Am. Sci*. **2010**, *6*, 123-128.

(120) Chitwood, D. J. Phytochemical based strategies for nematode control. *Annu. Rev. Phytopathol*. **2002**, *40*, 221-249.

(121) Atkinson, N. J., Dew, T. P., Orfila, C., Urwin, P. E. Influence of combined biotic and abiotic stress on nutritional quality parameters in tomato (*Solanum lycopersicum*). *J. Agric. Food Chem.* **2011**, *59*, 9673-9682.

(122) Khan, M. R., Haque, Z. Soil application of *Pseudomonas fluorescens* and *Trichoderma harzianum* reduces root-knot nematode, *Meloidogyne incognita*, on tobacco. *Phytopathol. Mediterr*. **2011**, *50*, 257-266.

(123) Wuyts, N., Swennen, R., De Waele, D. Effects of plant phenylpropanoi d pathway products and selected terpenoids and alkaloids on the behaviour of the plant-parasitic nematodes *Radopholus similis*, *Pratylenchus penetrans* and *Meloidogyne incognita*. *Nematology,* **2006**, *8*, 89-101.

(124) Pandey, R., Kalra, A. Chemic al activators: A novel and sustainable management approach for *Meloidogyne incognita* (Kofoid and White) Chitwood in *Chamomilla recutita* L. *Arch. Phytopathol*. *Plant Prot*. **2005**, *38*, 107-1110.

(125) Khoshkhoo, N., Hedin, P. A., McCarty, J. C. Effects of bioregulators on the terpenoid aldehydes in root-knot nematode infected cotton plants. *J. Agric. Food Chem*. **1993**, *41*, 2442 -2446.

(126) El-Rokiek, K., El-Nagdi, W. Dual effects of leaf extracts of *Eucalyptus citriodora* on controlling purslane and root-knot nematode in sunflower. *J. Plant Prot. Res*. **2011**, *51*, 121-129.

(127) Shazaukat, S. S., Siddiqui, I. A., Ali, N. I., Ali, S. A., Khan, G. H. Nematicidal and allelopathic responses of Lantana camara root extract. *Phytopathol. Mediterr*. **2003**, *42* ,71-78.

(128) Noweer, E. M.; Dawood, M. G. Efficiency of propolis extract on faba bean plants and its role against nematode infection. *Commun. Agric. Appl. Biol. Sci*. **2009**, *74*, 593−603.

(129) 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.

(130) Battle I., Tous J. Carob tree. Rome, Italy: *International Plant Genetic Resources Institute.* **1997**.

(131) Custódio, L., Carneiro, F. M. and Romano, A. Microsporogenesis and another culture in carob tree (*Ceratonia siliqua* L). *Sci. Hort*. **2005**, *104*, 65-77.

(132) Eman M. S. and Awlya O. F. A. Substituting of Cacao by Carob Pod Powder In Milk Chocolate Manufacturing. *Aust. J. Basic Appl. Sci*, **2012**, *6* (3), 572-578.

(133) Avallone, R., Plessi, M., Baraldi, M., Monzani, A. Determination of chemical composition of carob (*Ceratonia siliqua*): Protein, fat, carbohydrates, and tannins. *J. Food Compos. Anal*. **1997**, *10*, 166-172.

(134) Baytop, T. Therapy with Medicinal Plants in Turkey (Past and Present). Publications of the Istanbul University: Istanbul, **1984**; Vol. 3255.

(135) El Hajaji, H., Lachkar, N., Alaoui, K., Cherrah, Y., Farah, A., Ennabili, A., El Bali, B., Lachkar, M. Antioxidant properties and total phenolic content of three varieties of carob tree leaves from Morocco. *Rec. Nat. Prod*. **2010**, *4* (4),193-204.

(136) Amico, F. P., Sorce, E. G. Medical plants and phytotherapy in Mussomeli area (Caltanisetta, Sicily, Italy). *Fitoterapia*. **1997**, *68*, 143-159.

(137) Santos, M., Rodrigus, A., Teixeira, J. A. Production of dextran and fructose fromcarob pod extract and cheese whey by Leuconostoc mesenteroides NRRL B512(f). *Biochem. Eng. J.* **2005**, *25*, 1-6.

(138) Angioni, A., Barra, A., Cereti, E., Barile, D., Coisson, J. D., Arlorio, M., Dessi, S., Coroneo, V., Cabras, P. Chemical composition, plant genetic of essential oil of *Rosmarinus officinalis* L. *J. Agric. Food Chem*. **2004**, *52* , 3530-3535.

(139) Zygadlo, J. A., Guzman, C. A., Grosso, N. R. Antifungal properties of the leaf oils of *Tagetes minuta* L. and *T. filifolia* Lag. *J. Essent. Oil Res*. **1994**, *6*, 617-621.

(140) Corsi, L., Corsi, R., Avallone, F., Cosenza, F., Farina, C., Baraldi, M. Antiproliferative effects of *Ceratonia siliqua* L. on mouse hepatocellular carcinoma cell line. *Fitoterapia*. **2002**, *73*, 674-684.

(141) Akiyama, H., Fujii, K., Yamasaki, O., Oono, T., Iwatsuki, K. Antibacterial action of several tannin against *Staphylococcus aureus*. J. *Antimicrob. Chemother*. **2001**, *48*, 487-491.

(142) Anchana, C., Aphiwat, T., Jeremy, D. K., Nuansri, R. Antimicrobial gallic acid from *Caesalpinia mimosoides Lamk. Food Chem.* **2007**, *100*, 1044-1048.

(143) Panizzi, L., Caponi, C., Catalano, S., Ciono, P. L., Morelli, I. In vitro antimicrobial activity of extracts and isolated constituents of *Rubus ulmifolius*. *J. Ethnopharmacol*. **2002**, *79*, 165-168.

(144) Daglia, M. Polyphenols as antimicrobial agents. *Curr. Opin. Biotechnol*. **2012**, *23*, 174-181.

(145) Belguith, H., Kthiri, F., Chati, A., Abu Sofah, A., Ben Hamida, J., Landouls, A. Inhibitory effect of aqueous garlic extract (*Allium sativum*) on some isolated Salmonella serovars. Afr. *J. Microbiol. Res.* **2010**, *4* (5), 328-338.

(146) Lin, Y. T., Know, Y. I., Labbe, R. G., Shetty, K. Inhibition of *Helicobacter pylori* and associated urease by oregano and cranberry phytochimical synergies. *Appl. Environn. Microbiol.* **2005**, *71*, 5672-5678.

(147) Upson, T. M., & Andrews, S. The Genus Lavandula. London, UK: The Royal Botanical Gardens. **2004**.

(148) González-Coloma, A., Martín-Benito, D., Mohamed, N., García-Vallejo, M. C., & Soria, A. C. Antiffedant effects and chemical composition of essential oils from different populations of *Lavandula luisieri* L. *Biochem Syst Ecol*. **2006**, *34*, 609-616.

(149) González-Coloma, A., Delgado, F., Rodilla, J. M., Silva, L., Sanz, J., & Burillo, J. Chemical and biological profiles of *Lavandula luisieri* essential oils from western Iberia Peninsula populations. *Biochem Syst Ecol*. **2011**, *39*, 1-8.

(150) Barbosa, P., Lima, A. S., Vieira, P., Dias, L. S., Tinoco, M. T., Barroso, J. G., et al. Nematicidal activity of essential oils and volatiles derived from Portuguese aromatic flora against the pinewood nematodes, *Bursaphelenchus xylophilus*. *J. Nematol*. **2010**, *42*, 8-16.

(151) Roller, S., Ernest, B., & Buckle, J. The antimicrobial activity of high-necrodane and other lavender oils on methicillin-sensitive and resistant *Staphylococcus aureus* (MSSA and MRSA). *J Altern Complem Med*. **2009**, *15*, 275-279.

(152) Gonzalez-Coloma, A., Sainz, P., Olmeda, S., Burillo, J., Sanz, J. Umpierrez, M. L., Rossini. C. Desarrollo de métodos de bioensayos con garrapatas aplicados a la detección de potenciales bioplaguicidas botánicos. En: *Productos Naturales contra parásitos externos del ganado bovino y ovino, tales como mosca de los cuernos y garrapatas*, 2013, Pp. 56-69. Eds.

F. Echeverri, C. Rossini. Ediciones de la Universidad de Magallanes, Punta Arenas, Chile. ISBN 978-956-7189-52-6.

(153) Libran, C. M., Moro. A., Zalacain, A., Molina, A., Carmona, M., Berruga, M. I. Potential application of aromatic plant extracts to prevent cheese blowing. *World J Microbiol Biotechnol*, **2013**, *29*, 1179-1188.

(154) Gomes, G. A., Monteiro, C. M. O., Julião, L. D. S., Maturano, R., Senra, T. O. S., Zeringóta, V., Calmon, F., Matos, R. D. S., Daemon, E., Carvalho, M. G. D. Acaricidal activity of essential oil from Lippia sidoides on unengorged larvae and nymphs of *Rhipicephalus sanguineus* (Acari: Ixodidae) and *Amblyomma cajennense* (Acari: Ixodidae) *Exp. Parasitol*, **2014**, *137* (1), pp. 41-45.

(155) Olmeda, A. S., Perez, J. L., Martin-Hernandez, R., Torrente, M., Valcarcel, F. Toxicity of Oxalic Acid Against Adult *Hyalomma lusitanicum* Ticks (Ixodida: Ixodidae) in Laboratory Conditions: LD50. *Entomological Society of America*, **2008**, *45*(4), 715-719.