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Master's dissertation
Plant Molecular Biology, Biotechnology and
Bioentrepreneurship

Work supervised by
Professora Doutora Maria Teresa da Silva Craveiro
Martins de Almeida
Doutora Maria Clara Almeida Vieira dos Santos

janeiro de 2022

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# Controlo de nemátodes-das-galhas-radiculares, *Meloidogyne incognita*, por bioprodutos vegetais

## Resumo

Os nemátodes-das-galhas-radiculares (NGR), *Meloidogyne* spp., são fitoparasitas responsáveis pela redução do rendimento e qualidade de culturas. Diferentes abordagens têm sido implementadas para controlar as suas populações. Vários extratos botânicos e óleos essenciais (OEs) foram referidos pela atividade nematodicida contra NGR e são cada vez mais considerados para uso em programas integrados de gestão de pragas e doenças (IPM). Neste estudo, foram selecionadas diversas espécies vegetais pela disponibilidade e potencial uso como pesticidas botânicos. A atividade nematodicida de extratos hidroetanólicos (EHEs) e OEs de *Cymbopogon citratus*, *Mentha aquatica*, *M. piperita*, *M. pulegium*, *Satureja montana* e *Thymus vulgaris* foi avaliada sobre a mortalidade e infetividade de jovens do 2º estádio (J2) de *Meloidogyne incognita*. Os EHEs mais eficazes foram os de *C. citratus*, *Mentha. aquatica* e *M. pulegium*, com valores de $LC_{50}$ de 8,63, 5,98 e 5,32 mg/mL (p<0,05). Os OEs mais eficazes foram os de *C. citratus*, *S. montana* e *T. vulgaris*, os quais induziram cerca de 100% de mortalidade de J2 após 24h, à concentração de 2000 ppm. Com base nos resultados de mortalidade obtidos com EHEs, foram selecionados os EHEs e OEs de *C. citratus*, *M. aquatica* e *M. pulegium* para testar o seu efeito na infetividade de J2, em ensaios de micro-vaso. Após exposição de J2 a concentrações selecionadas durante 48h, estes foram inoculados em plântulas de tomateiro cv. Tiny Tim com 10 dias. Sete dias após a inoculação, os J2 no interior das raízes foram corados com fucsina ácida, e contabilizados. Após exposição a $LC_{50}$ de EHEs de *C. citratus*, *M. aquatica* e *M. pulegium*, houve uma redução de 66,7, 60,9 e 69% de J2. Após exposição a concentrações selecionadas de OEs de *C. citratus* (250 ppm), *M. aquatica* e *M. pulegium* (2000 ppm), houve uma redução na infetividade de 87,5, 77,8 e 84,4%, respetivamente.

O efeito do OE de *C. citratus* encapsulado em microcápsulas de queratina na mortalidade de J2 foi investigado, após 48h. Verificou-se que o OE encapsulado afetou um número superior de J2 do que na sua formulação livre (p>0.05). Foram obtidas imagens por microscopia de fluorescência que indicaram a internalização do OE pelos J2; além disso, os J2 que morreram após incubação com o OE encapsulado apresentavam alterações morfológicas do intestino.

Os resultados obtidos parecem indicar que os EHEs de *C. citratus*, *M. aquatica* e *M. pulegium* e OEs de *C. citratus*, *M. pulegium*, *S. montana* e *T. vulgaris* têm potencial como agentes nematodicidas e como novas formulações que possam ser incorporadas em programas IPM.

**Palavras-chave:** ação anti-nemátode; encapsulação; extratos botânicos; gestão; óleos essenciais

# Control of the root-knot nematode, *Meloidogyne incognita*, using plant bioproducts

## Abstract

Root-knot nematodes (RKN), *Meloidogyne* spp. are plant pathogens responsible for yield reduction and quality loss in crops. Several approaches have been implemented in order to control its populations. Various botanical extracts, and essential oils (EOs), have been reported to have nematicidal activity against RKN and are increasingly being considered for use in integrated pest management (IPM) programs. In this work, various plant species were selected based on their availability and potential use as botanical pesticides. The nematicidal activity of hydroethanolic extracts (HEEs) and EOs of *Cymbopogon citratus*, *Mentha aquatica*, *M. piperita*, *M. pulegium*, *Satureja montana* and *Thymus vulgaris* was evaluated on their effect on mortality and infectivity on second-stage juveniles (J2) of *Meloidogyne incognita.* The most effective HEEs were of *C. citratus*, *Mentha aquatica and M. pulegium*, with $LC_{50}$ values of 8.63, 5.98 and 5.32 mg/mL ($p<0.05$). The most effective EOs were of *C. citratus*, *S. montana* and *T. vulgaris*, which induced the mortality of approximately 100% of J2 after 24h of exposure to the concentration of 2000 ppm. Based on the mortality results obtained with HEEs, the *C. citratus*, *M. aquatica* and *M. pulegium* HEEs and EOs were selected to test their effect on J2 infectivity, in micro-pot assays. After J2 exposure to selected concentrations for 48h, they were inoculated on 10-day old tomato cv. Tiny Tim seedlings. Seven days after inoculation, J2 inside the roots were stained with acid fuchsin, and counted. After exposure to $LC_{50}$ of *C. citratus*, *M. aquatica* and *M. pulegium* HEEs, there was a reduction of 66.7, 60.9 and 69% of J2. After exposure to concentrations of *C. citratus* (250 ppm), *M. aquatica* and *M. pulegium* (2000 ppm) EOs there was a reduction in infectivity of 87.5, 77.8 and 84.4%, respectively.

The effect of *C. citratus* EO encapsulated in keratin microcapsules on the mortality of J2 was investigated, after 48h. The encapsulated EO affected a superior number of J2, than in its free formulation ($p>0.05$). Images were obtained by fluorescence microscopy, which indicated the internalization of the EO by the J2, and the J2 that died after incubation with the encapsulated EO presented morphological changes in the intestine

The results obtained seem to indicate that the HEEs of *C. citratus*, *M. aquatica* and *M. pulegium* and EOs of *C. citratus*, *M. pulegium*, *S. montana* and *T. vulgaris* have potential as nematicidal agents and as new efficient formulations that can be incorporated in IPM programs.

**Keywords:** anti-nematode action; botanical extracts; encapsulation; essential oils; management.

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# I. Introduction

Plant parasitic nematodes (PPN) are obligate plant pathogens that represent a significant constraint on global food production. They can be found in every continent, associated with nearly every important agricultural crop, and are considered one of the most damaging biotic stresses on crops (Fuller *et al.*, 2008). These nematodes are responsible for yield reduction and quality loss in crops, by causing lesions in plants (Karssen and Moens, 2006). The degree of damage and yield suppression increases in synergy with infection by other soil-borne pathogens such as fungi, bacteria and virus. In addition, soil and climatic conditions also influence the threshold population density, above which measurable damage occurs (Back *et al.*, 2002). Assessing PPN impact is also difficult because damage resulting from nematode infection is often less obvious than that caused by many other pests or diseases. The economic losses due to PPN are not only confined to yield reductions, but also to an increase on production costs for farmers, such as waste of irrigation, water and fertilizers (Wesemael *et al.*, 2011).

## 1. Root-knot nematodes (*Meloidogyne* spp.)

In 2013 PPN were ranked based on scientific and economic importance and root-knot nematodes (RKN), *Meloidogyne* spp., were considered the most important genera. Root-knot nematodes are sedentary endoparasites that can be found worldwide, have a wide host range and present rapid population growth, mainly due to the completion of several generations during a single growing season, and because females are capable of producing up to a thousand eggs (Jones *et al.*, 2013). Infection by RKN causes deformation of the root system, resulting in reduced uptake of nutrients and water, and translocation of solutes (Karssen and Moens, 2006)

The lifecycle of RKN varies from three weeks to several months depending on environmental factors, such as temperature, moisture and availability of suitable host. Eggs are laid in a gelatinous matrix produced by the adult female. Embryogenesis is followed by the first moult to the infective second-stage juvenile (J2). In the soil, the J2 search for a host plant and penetrate directly behind the root tip, and migrate intercellularly, eventually reaching the root vascular cylinder where they create specialized feeding sites called giant cells (Perry *et al.*, 2009; Jones *et al.*, 2013). The nematode becomes sedentary and moults to the third-stage juvenile (J3), then to the fourth-stage juvenile (J4) and finally to the adult stage. Males exit the roots without harming the host. If the juvenile differentiates into an adult female, it will remain sedentary and continue to feed and swell, eventually becoming pyriform (pear-shaped). The female posterior end will break through the

epidermis of the root where it will deposit the eggs within a protective gelatinous matrix outside her body (Perry *et al.*, 2009). More than 100 species of *Meloidogyne* genus have been described (Bernard *et al.*, 2017). Around the world, *M. arenaria, M. hapla, M. incognita* and *M. javanica* are serious ubiquitous pests being considered as "major" species (Rusinque *et al.*, 2017). *Meloidogyne incognita* has been considered as one of the most prevalent species in warmer conditions of southern Europe, and also in glasshouses in northern Europe and is able to infect almost all cultivated plants, and exponentially increase population levels due to rapid parthenogenetic multiplication, meaning that the females reproduce without the presence of a male (Wesemael *et al.*, 2011). Its genome was sequenced in 2008 in order to provide insights into the adaptations required by metazoans to successfully parasitize and counter defenses of immunocompetent plants (Abad *et al.*, 2008).

## 2. Root-knot nematode control

The main objective of RKN control is to keep nematode populations below threshold levels, and several approaches have been implemented, such as: chemical control, cultural control and biological control. However, management of RKN under intensive crop cultivation systems is hard due to their resilience and ubiquity, and no single management strategy has proven successful at eliminating RKN, so it relies mainly on the use of integrated pest management (IPM) programs (Rusinque *et al.*, 2017).

The major control method of RKN is based on the use of chemical nematicides, since these are effective and fast acting (Taimoor and Shahina, 2018). Fumigant nematicides are considered more effective in controlling RKN and in increasing crop yield than non-fumigant nematicides, due to their broad-spectrum activity (Netscher and Sikora, 1990). The downside is that they are expensive, can accumulate in plant tissues, and are environmentally hazardous and toxic causing environmental pollution, and endangering the life of animals and humans. As a result, many of the chemical nematicides have been banned (91/414/EEC) or are under evaluation (2009/1107/EU) by strict legislations in European Union countries, thus reducing control options. Hence, there is a global interest in developing less toxic and more environment-friendly alternatives.

Cultural practices are the most economical means of managing insect-pests and diseases, including nematode problems (Ghule *et al.*, 2014). Cultural control includes strategies such as: crop rotation with, cover crops, soil solarization, trap cropping, biofumigation, and use of resistant

cultivars. Crop rotation is one of the oldest and most important methods for managing nematodes in annual crops. However, this strategy does not allow intensive agricultural practices and resistant or tolerant crops may not be an economically attractive option. Additionally, crop rotation may further increase selection pressure for virulent populations. Furthermore, the success of this control strategy is limited because of the wide host ranges of most RKN species and the frequent occurrence of infestations induced by more than one species (Wesemael *et al.*, 2011). Cover cropping has been used in IPM protocols to reduce PPN incidence and replenish soil nutrient levels. Cover crops help improve soil fertility, soil structure, decrease soil erosion, and suppress weeds, insects, nematodes, and other plant pathogens (Gill and Mcsorley, 2011). The major limitation is the wide host range of RKN (Van der Putten *et al.*, 2006). Solarization is usually performed in mid-summer to maximize soil-heating effects. The soil is covered with plastic film for at least 2 weeks, with the intent to destroy egg masses, reducing the population density of RKN, but it is only viable in regions where sufficient solar energy is available for long periods of time (Mediterranean countries). This method is also only effective up to a depth of 20 cm in the soil, and as J2 are able to migrate deep into the soil escaping from high temperatures (Bello *et al.*, 2000). Trap crops are plants used to suppress or directly control nematode populations. These must be good hosts to allow the easy infection of the nematode species. After RKN invade the roots and establish a feeding site, the females become sedentary and before the nematodes complete their life cycle, the crop is destroyed, in order to destroy the root systems and all the nematodes trapped within. This way a new soil infestation is avoided, reducing the nematode population. Some examples of trap crops are tomatoes and carrots. This technique implies the destruction of plants with no economic return (Sacchi *et al.*, 2021). Biofumigation consists in the action of volatile substances produced in the bio-decomposition of organic matter. Some examples of biofumigants successfully used in crops are cow manure, and crop remains from rice and ornamental gardens (Bello *et al.*, 2000). When biofumigation is used in combination with other techniques, such as solarization, it is a very useful and efficient method to control RKN in IPM programs (Medina-Míguez, 2002). However, biofumigants are slow acting upon target organisms, which requires the application of organic amendments at high rates in order to have an effect on nematode populations (Van der Putten *et al.*, 2006).

Plant resistance is probably the most environmentally safe method to control RKN (Ibrahim *et al.*, 2016). It can be, either naturally occurring and transferred to crop cultivars through conventional breeding methods with wild relatives, or engineered through molecular techniques. The most widely

used and investigated approach is the integration of *Mi* gene, which was introgressed from the wild tomato species *Lycopersicon peruvianum* (L.) Mill, however, resistance mediated by *Mi* gene is lost at soil temperatures above 28 °C, and races breaking resistance have been found in *M. incognita* (Dropkin, 1969; Starr *et al.*, 2002). Resistant cultivars are also only available for a limited number of crops and PPN, and it has been stated that virulence against *Mi* gene can develop in some cases after just as few as five plantings (Noling, 2000).

Biological control is based on the use of biopesticides, such as antagonistic agents and botanical extracts, to control or reduce population densities of pathogens and pests. Parasitism and predation are the main mechanisms of action of these antagonists, but antibiosis, induced resistance of the host plant, and direct competition for space and limited resources, are some other factors that may lead to the reduction of the infection level (Wiratno *et al.*, 2020). However, biological control agents will generally provide too little control to be effective alone, as they are required in large amounts, and tend to be difficult to establish in the field (Paul *et al.*, 2015).


## 3. Botanical extracts and essential oils with nematicidal activity

Plants can be used as pesticides themselves, or they can serve as model compounds for the development of chemically synthesized, easily biodegradable derivatives, with low plant and human toxicity (Chitwood, 2002). The presence of bioactive compounds with nematicidal properties in a wide range of plants, acting as repellents, inducing paralysis, reducing hatch or even causing death, presents a potential alternative to the use of synthetic nematicides (Stamp, 2003; Renčo *et al.*, 2014). Several secondary metabolites from plant extracts and essential oils are among these compounds and a substantial number in several major classes have been reported to show nematicidal activity, with the most important ones being alkaloids, monoterpenoids, flavonoids, diterpenoids, and polyphenols (Andrés *et al.*, 2012, Renčo *et al.*, 2014). The recognition of the importance of plants secondary metabolites in terms of resistance to pests has increased, and so, plant extracts and essential oils (EOs) are increasingly being considered for use in IPM programs (Wiratno *et al.*, 2020)

Several botanical extracts, and EOs have been reported to have nematicidal activity against *Meloidogyne* spp. Some examples of commercial biological nematicides derived from plant material, with the ability to suppress RKN in an ecologically sustainable manner, are Dazitol®

(capasaicin and essential oil of mustard) and Nemakill® (cinnamon oil, clove oil, and thyme oil mixture) (Watson and Desaeger., 2019).

Different plant extracts from aromatic plants, in the solid state or as aqueous solutions have been tested against *M. incognita* and had significant effects on the hatching, and mortality of J2 after 24h of exposure to low concentrations (1-100 mg/L). The extract of garlic (*Allium sativum* L.) significantly reduced hatching activity to below 8% at the concentration of 1 mg/L, and the extract of *Foeniculum vulgare* Mill. was toxic to J2, with an obtained $LC_{50}$ of 43 mg/L (Ibrahim *et al.*, 2006; Caboni *et al.*, 2013). There are also examples, where powder and extracts of different plant parts have been used for the management of RKN. Neem (*Azadirachta indica* A. Juss.) is the best-known example that acts by releasing pre-formed nematicidal constituents into soil (Prakash *et al.*, 2014).The effects of methanolic or ethanolic extracts of various plants (mainly the aerial parts) against *M. incognita* have also been investigated, and some ethanolic extracts, like the extract of tobacco leaves (*Nicotiana tabacum* L.) and clove buds (*Syzygium aromaticum* L.) showed high nematicidal activity against J2 after 24h of exposure, with $LC_{50}$ values that were 5-10 times lower than the $LC_{50}$ of the synthetic pesticides chlorpyrifos, carbosulfan and deltamethrin. (Wiratno *et al.*, 2009; Pavaraj *et al.*, 2012).

Essential oils from various plants have nematicidal properties due to the presence of secondary metabolites, such as carvacrol and thymol (Oka *et al.*, 2000), which have been shown to completely inhibit hatching of *M. incognita* eggs at low concentrations (2, 4 mg/L) (Ibrahim *et al.*, 2006). Among the several plants tested in direct contact assays, the EOs of aromatic plants of the Poaceae and Lamiaceae families, from the *Cymbopogon* and *Mentha* genus, for example, have been widely studied in regards to nematicidal activity towards *Meloidogyne incognita* and *M. javanica* (Oka *et al.*, 2000; Ntalli *et al.*, 2010; Gupta *et al.*, 2011; Andrés *et al.*, 2012; Caboni *et al.*, 2013). Essential oils are generally more complex and have more concentrated active compounds than tinctures or extracts, and investigations are focused mainly on the nematicidal properties of EOs (Amora *et al.*, 2017).

This study is part of a project (POCI-01-0145-FEDER-031131) in which different plant species were selected based on their availability and potential use as botanical pesticide against different organisms.

### 3.1 *Cymbopogon citratus*



**Figure 1.** Wild lemongrass, *Cymbopogon citratus.* (Source: Zebua, 2020)

Plants of the genus *Cymbopogon* are widely distributed across all continents and *Cymbopgon. citratus* (DC.) Stapf (Fig. 1), commonly known as wild lemongrass, is one the most important in terms of EO production (Avoseh *et al.*, 2015). This plant is used in traditional and modern medicine in many countries for a wide range of applications, based on its antibacterial, antifungal, antiprotozoal, anti- inflammatory, antioxidant, cardioprotective, antitussive and antiseptic activities. Its leaves are used to make tea as a homemade remedy for cough, flu, gingivitis, digestive problems and stomachache. In addition to its therapeutic uses, aqueous extract and essential oils from this plant are also used in the food, perfume and cosmetics industries, due to their typical lemon and rose-like aroma. The EO of *C. citratus* contains various monoterpenes, with citral being the most abundant (65%–85%) (Ekpenyong *et al.*, 2015). Citral is a mixture of terpenoids neral and geranial, and is the main component responsible for the typical aroma (Ekpenyong *et al.*, 2014). The nematicidal activity of *C. citratus* EO is also mainly attributed to the content of citral (Barros *et al.*, 2020).

It has been shown by different authors that the use of leaf extracts of lemongrass can be of economic benefit in the management of nematode pests, since it has been proven to be effective in controlling *M. incognita*, by reducing the number of formed galls (Corbani and Mazzonetto., 2013; Izuogu *et al.*, 2015). The nematostatic effect of *C. citratus* EO has been demonstrated, after *C. citratus* EO (1000 ppm, dissolved in ethanol – 10% vol/vol and Tween 20 – 0.3% vol/vol) affected the mobility of 69.3% of J2 of *M. javanica*, after 48h (Oka *et al.*, 2000). Different studies have also described the mortality that EOs of *Cymbopogon* grasses caused to J2 of *M. incognita*.

The mortality of *M. incognita* J2 was reported after *C. citratus* EO (1:2 in Texapon N-40) caused the mortality of 63% of J2 at the concentration of 625 ppm, and 100% at the concentration of 1250 ppm, after 16 days of exposure (Moreira *et al.*, 2009). In a different study, the EO (1000 ppm, dissolved in Tween 80 – 0.5% vol/vol) caused the mortality of 100% of J2 and the mortality of approximately 70% of J2 at the concentration of 120 ppm, after 48h of exposure (Gupta *et al.*, 2011). Similarly, *C. citratus* EO (dissolved in 1% Tween 80), was tested on *M. incognita* J2 at different concentrations, and a LC50 value of 166 ppm was obtained after 48h (Barros *et al.*, 2020).

## 3.2 *Mentha* species



**Figure 2.** *Mentha* species: water mint, *Mentha aquatica* (A), peppermint, *Mentha piperita* (B) and pennyroyal mint, *Mentha pulegium* (C). (Source: Newman, 2020; Bolshakov, 2019; Barros, 2012)

The genus *Mentha* (Lamiaceae) comprises several aromatic species, which are cultivated around the world due to their distinct aroma and commercial value. Fresh and dried plant material, raw extracts and essential oils of mint plants are used for preparation of herbal teas and as supplements to various commercial products including cosmetics, foods and drugs (Mahendran and Rahman, 2020). In addition to the traditional flavoring uses, *Mentha* spp. extracts and EOs are well recognized for their folk medicinal uses due to their anti-inflammatory, antiemetic, diaphoretic, antispasmodic, anti-catarrhal, analgesic, antibacterial and antioxidant properties. The traditional biological and pharmacological attributes can be linked to the high content of secondary metabolites such as terpenoids, alcohols, rosmarinic acid, and antioxidant phenolics among others (Anwar *et al.*, 2019).

*Mentha aquatica* L. (Fig. 2A) is used in beverages, salads or cooked foods. Besides the food applications, water mint is also been consumed as tea, and used in traditional medicine for the

treatment of external inflammation (mouth or throat problems) and in inflammation-related diseases, such as rheumatism (Anwar *et al.*, 2019).

Among the diversity of plants, *Mentha piperita* L. (Fig. 2B) is one of the herbs most widely used worldwide, with a long history of safe use in medicinal preparations. Peppermint is among the most popular single ingredient herbal teas and its oil is one of the most popular widely used essential oils. (Mahendran and Rahman., 2020).

*Mentha pulegium* L. (Fig. 2C) is a traditional culinary herb, folk remedy, and abortificient. Pennyroyal mint leaves, both fresh and dried, are especially noted for repelling insects. Its oil is poisonous, yet it is used to make herbal teas, which, although not proven to be dangerous to healthy adults in small doses, is not recommended, due to its known toxicity to the liver (Miraj *et al.*, 2016).

The nematostatic activity of water extracts, methanolic extracts and EOs of *M. piperita*, *M. pulegium*, and *M. spicata*, has been previously evaluated. The methanolic extracts were not biologically active, and the water extracts exhibited nematostatic activity against *Meloidogyne incognita* J2 after 72h of exposure ($EC_{50}$ values of: 1.00, 0.75, and 0.30 mg/mL, respectively) (Caboni *et al.*, 2013). The nematostatic and nematicidal activity of *Mentha piperita*, *M. pulegium* and *M. spicata* EOs on RKN have also been evaluated by various authors (Table 1). In a study with *Meloidogyne javanica*, *Mentha piperita* and *M. spicata* EOs affected the mobility of 71.8 and 100% of J2, after 48h (Oka *et al.*, 2000). The nematostatic effect of *M. spicata* EO against *Meloidogyne incognita* was reported, with an $EC_{50}$ of 358 ppm, after 72h (Caboni *et al.*, 2013). The effect of *Mentha pulegium* EO against *Meloidogyne javanica* J2 was also reported after the EO exhibited nematostatic activity, with an $EC_{50}$ value of 3890, after 48h (Ntalli *et al.*, 2010). In a work with *Meloidogyne incognita*, *Mentha piperita* and *M. spicata* EOs induced the mortality of 35 and 50% of J2, after 24h (Pandey *et al.*, 2000). In a similar study with *Meloidogyne javanica*, *Mentha spicata* EO induced 100% mortality, after 12h (Andrés *et al.*, 2012). Furthermore, it has also been reported that *M. piperita* EO induced 99.1% mortality of *Meloidogyne javanica* J2, after 24h (Amora et., al 2017).

**Table 1.** Reported effects of *Mentha* spp. essential oils on the mobility and mortality of second-stage juveniles (J2) of *Meloidogyne incognita* and of *M. javanica.*

| Plant species | *Meloidogyne* spp. | Time (h) | Concentration tested | Assay | EC50/ LC50 (ppm) | Effect (%) | Solvents | Reference |
|---|---|---|---|---|---|---|---|---|
| *Mentha piperita* | *M. javanica* | 48 | 1000 | mobility | - | 71.8 | Ethanol (10% vol/vol); Tween 20 (0.3% vol/vol) | Oka *et al.*, 2000 |
| *M. spicata* | *M. javanica* | 48 | 1000 | mobility | - | 100 | | |
| *M.piperita* | *M. incognita* | 48 | 1000 | mortality | - | 35 | DMSO (10% vol/vol); Tween 80 (4.5% vol/vol) | Pandey *et al.*, 2000 |
| *M. spicata* | *M. incognita* | 48 | 1000 | mortality | - | 50 | | |
| *M pulegium* | *M. javanica* | 24 | - | mobility | 4520 | - | Tween 20 (0.3% vol/vol) | Ntalli *et al.*, 2010 |
| | | 96 | - | | 3150 | - | | |
| *M. piperita* | *M. javanica* | 12 | 1000 | mortality | - | a | DMSO; Tween 20 (0.5% vol/vol) | Andrés *et al.*, 2012 |
| *M. spicata* | *M. javanica* | 12 | 1000 | mortality | 293 | 100 | | |
| *M. piperita* | *M. incognita* | 72 | 1500 | mobility | a | - | Tween 20 (0.3% vol/vol) | Caboni *et al.*, 2013 |
| *M. pulegium* | *M. incognita* | 72 | 1500 | mobility | a | - | | |
| *M. spicata* | *M. incognita* | 72 | 1500 | mobility | 358 | - | | |
| *M. piperita* | *M. javanica* | 24 | 2500 | mortality | - | 99.1 | DMSO (2% vol/vol) | Amora *et al.*, 2017 |

a - Essential oil was not biologically active

- Missing data indicate lack of information

## 3.3 *Satureja montana*



**Figure 3.** Wintor savory, *Satureja montana.* (Source: Wirth, 2021)

The genus *Satureja* (Lamiaceae) contains around 200 species of aromatic herbs and shrubs. *Satureja montana* L. (Fig. 3) is a perennial small herb native to arid, sunny, and rocky regions in the Mediterranean area, but it is cultivated throughout Europe. It is an aromatic plant widely used as a spice and as a traditional medicinal herb (Hudz *et al.*, 2020). Ethanolic extracts and EOs of

*S. montana* revealed antioxidant and antimicrobial properties. The leaves, flowers, and stems are used for herbal tea and, in traditional medicine, to treat various ailments, due to several activities such as digestive, expectorant and laxative, among others. Furthermore, it is often used in Mediterranean cooking preparations and even as natural antibacterial agent in food packaging (Maccelli *et al.*, 2019). The nematicidal activity of *S. montana* EO can be attributed to its content in carvacrol and thymol (Andrés *et al.*, 2012).

*Satureja montana* EO obtained from flowers and leaves (1000 ppm, dissolved in water with DMSO and Tween 20 – 0.5% vol/vol) has been shown to possess nematicidal activity against *M. javanica* J2, after 12h, with a LC$_{50}$ of 41 ppm (Andrés *et al.*, 2012). In a study with *M. chitwoodi,* two EOs of *S. montana* displayed hatching inhibitions of over 90%, after 72h of exposure to the concentration of 2000 ppm, and were further tested at the concentration 125 ppm, presenting EC$_{50}$ values of 61 and 33 ppm (Faria *et al.*, 2016).

## 3.4 *Thymus vulgaris*



**Figure 4.** Thyme, *Thymus vulgaris.* (Source: Gort, 2008)

The genus *Thymus* (Lamiaceae), thyme, comprises over 350 aromatic plant species with quite different botanical characteristics and broad chemical heterogeneity. *Thymus* spp. are used as drug because they possess a wide range of biological activities including antimicrobial, antibacterial, antispasmodic, antifungal and antioxidant properties due to the presence of flavonoids, polyphenol and phenolic acids (Hosseinzadeh *et al.*, 2015). Its nematicidal activity can be explained by its content in carvacrol and thymol (Andrés *et al.*, 2012). Among the different species, *Thymus vulgaris* L. (Fig. 4) is a flowering plant native to Southern Europe with a worldwide

distribution (Hosseinzadeh *et al.*, 2015). It is an important medicinal plant that has been used for centuries as spice, homemade remedy, drug, perfume and insecticide (Salehi *et al.*, 2018).

The undiluted aqueous extract of *T. vulgaris* shoots has been shown to affect 100% of *M. incognita* J2 in regards to mobility, after 72h (Korayem *et al.*, 1993). Several studies have also reported the effects of the EO on the mobility and mortality of *M. incognita* and *M. javanica* (Table 2). The nematostatic activity of *T. vulgaris* EO was reported in a study with *M. javanica*, after the EO affected the mobility of 67.5% of J2, after 48h of exposure (Oka *et al.*, 2000). The effects of *T. vulgaris* EO on *M. incognita* J2 was noted, after the EO induced the mortality of approximately 70% of J2, after 48h (Barros *et al.*, 2019). In a work with *M. javanica*, *T. vulgaris* EO induced 100% mortality of J2, after 12h, and presented a LC$_{50}$ value of 224 ppm (Andrés *et al.*, 2012). Furthermore, it was also demonstrated in a different study, that *T. vulgaris* EO induced 99.4% mortality of *M. javanica* J2, after 24h of exposure (Amora et., al 2017).

Table 2. Reported effects of *Thymus vulgaris* essential oil on the mobility and mortality of second-stage juveniles (J2) of *Meloidogyne incognita* and of *M. javanica.*

| Plant species | Meloidogyne spp. | Time (h) | Concentration tested | Assay | EC50/ LC50 (ppm) | Effect (%) | Solvents | Reference |
|---|---|---|---|---|---|---|---|---|
| *Thymus vulgaris* | *M. javanica* | 48 | 1000 | mobility | - | 67.5 | Ethanol (10% vol/vol); Tween 20 (0.3%) | Oka *et al.*, 2000 |
| *T. vulgaris* | *M. javanica* | 12 | 1000 | mortality | 224 | 100 | DMSO; tween 20 (0.5% vol/vol) | Andrés *et al.*, 2012 |
| *T. vulgaris* | *M. javanica* | 24 | 2500 | mortality | - | 99.4 | DMSO (2% vol/vol) | Amora *et al.*, 2017 |
| *T. vulgaris* | *M. incognita* | 48 | 1000 | mortality | - | 70 | Tween 20 (1% vol/vol) | Barros *et al.*, 2019 |

- Missing values indicate lack of information

Botanical or plant-derived compounds show great promise as an alternative to chemical control because they have a specific mode of action, are eco-friendly, do not leave toxic residues in the edible plant parts, and can be prepared locally (Taye *et al.*, 2012). However, they may present limited persistence in field conditions, and the chemical composition of plant secondary metabolites varies considerably between plant species and varieties, level of maturity, extraction process, and even within the same variety from different geographic areas (Andrés *et al.*, 2012).

## 4. Encapsulated botanical extracts and essential oils (EOs) with nematicidal activity

Nanotechnology is an emerging area that poses a potential solution to the limited persistence of plant extracts and their bioactive compounds in field conditions, due to its long-lasting beneficial activity, and favorable safety profile (Iavicoli et al., 2017). It presents a new way to improve the efficiency of plant extracts or their biocidal compounds through the construction of nanotechnology-based agricultural systems such as drug carriers and controllable releasing systems (Nakamura et al., 2017).

By encapsulating compounds through a physical or chemical process, beads can be produced with size ranges from a few nm to a few mm (Devi et al., 2019). Some benefits of encapsulated formulations include: possibility of slow release of active ingredients, improved stability by preventing early degradation, and use of environmentally friendly components, such as nontoxic solvents and biodegradable natural matrices (Ashoka et al., 2017). The effectiveness of natural extracts may be enhanced, improving their efficiency, while reducing the amount used. There are many kinds of naturally-derived biomaterials, and among them keratin has emerged as a potential candidate, as its exploration as a biomaterial for controlled drug delivery has widely expanded, due to its biocompatibility, biodegradability, and natural abundance (Wang et al., 2016). Nanoemulsion of plant EOs is one of the best studied bionanopesticide delivery systems for pest management, since EOs present poor solubility in water and immobility in soil, meaning they are usually trapped in soil organic matter, making them less effective when applied for nematode control (Devi et al., 2019). The simplest use of nanomaterials against RKN is their direct application into the soil. Some nano-products or nanomaterials, such as nano-silver and nano-sulfur have already been used against RKN (Sabry, 2019). Silver nanoparticles (Ag-NPs) synthesized from extracts of Conyza dioscoridis L., Melia azedarach L., and Moringa oleifera Lam. showed increased nematicidal activity up to twofold against eggs and up to fivefold against J2 of Meloidogyne incognita when compared to crude leaf extracts (Abbassy et al., 2017). In another study with M. incognita, Ag-NP synthesized from Artemisia judaica L. extracts caused threefold increase in J2 mortality and twofold increase in egg hatch inhibition in comparison with crude leaf extracts (Soliman et al., 2017).

The long-term effects of encapsulated formulations on human health and environmental quality when applied in crop protection are still uncertain, but the prospects of encapsulation are bright for increasing the efficacy of biopesticides against phytopathogens (Devi et al., 2019).

## 5. Objectives

The main objective is to determine the activity and anti-nematode potential of hydroethanolic extracts (HEEs) and essential oils (EOs) of *Cymbopogon citratus*, *Mentha aquatica, M. piperita, M. pulegium, Satureja montana* and *Thymus vulgaris*, with the perspective of being effectively used as biopesticides for RKN management, more specifically:

i) to evaluate the effect of HEEs and EOs on the mortality of *Meloidogyne incognita* J2

ii) to assess the effect of selected HEEs and EOs on the infectivity of *M. incognita* J2

iii) to estimate the nematotoxic effect of a selected EO under an encapsulated formulation, on the mortality of *M. incognita* J2

# II. Materials and methods

## 1. *Meloidogyne incognita* isolate

The *M. incognita* isolate used throughout this work belongs to the collection of the Nematology group from the Center of Molecular and Environmental Biology (CBMA), University of Minho, and was originally isolated from potato roots of *Solanum tuberosum* L. cv. Asterix cultivated in a field located in Vale da Caparica, Setúbal (Esteves *et al.*, 2015).

In this study, *S. lycopersicum* L. tomato plants, cv. Tiny Tim were used to maintain and propagate the isolate. Seeds were left to germinate in the dark, in an incubator at 24 °C, inside a Petri dish with moist paper. After germination, and when they presented a small radicle, they were transferred to plastic pots (40 cm³), containing a mixture of autoclaved soil, vermiculite and sand, in equal proportions. The pots were transferred to an air-conditioned room with a 12-hour photoperiod, at a temperature ranging between 20-25 °C and relative humidity between 30-75%. The plants were watered daily with tap water. After 2 to 3 weeks, when the plants reached 2 pairs of true leaves, they were transferred to pots (400 cm³) filled with the same soil mixture. The plants were inoculated by distributing 10 egg masses hand-picked from infected roots, in 3 equidistant holes (3-4 cm deep) close to the stem. The plants were kept in an air-conditioned room in the conditions described above.

To obtain J2 for the bioassays, egg masses, collected from infected roots, were placed on sieves, constructed with a plastic ring (2.5 cm diameter; 0.9 cm height) and a nylon mesh (30 µm mesh aperture) attached at the bottom by an elastic band. Each sieve was placed on top of a plastic net grid, inside a Petri dish (60×15 mm), and 5 mL of autoclaved deionized water was added, in order to fill the Petri dishes halfway and submerge the bottom of the sieve. Petri dishes were incubated in the dark, at room temperature. The resulting suspension was collected every 72 h and the number of J2 was estimated by counting the number of J2 in three 1 ml replicates.

## 2. Effect of plant bioproducts on *M. incognita* second-stage juveniles (J2) mortality

The potential nematicidal activity of hydroethanolic extracts (HEEs) and essential oils (EOs) (Table 3), on J2 of *M. incognita* was tested by *in vitro* bioassays using the methodology described in Costa *et al.* (2003) with some modifications. The plant bioproducts used in this study were kindly provided by Prof. Manuel F. Ferreira, from the Faculty of Sciences of the University of Porto (FCUP) and are listed in Table 3. The lyophilized HEEs (obtained after maceration of grounded plant parts,

purchased from Ervital, with 70% ethanol without separation or fractionation) and the EOs were kept in the dark, in a desiccator, inside a cold room, at 4 °C until needed.

**Table 3.** Plant species and material used in the preparation of the hydroethanolic extracts (HEEs) and essential oils (EOs).

| Plant species | Common name | Plant material used for extraction | |
| | | HEE | EO |
|---|---|---|---|
| *Cymbopogon citratus* | wild lemongrass | leaves | leaves |
| *Mentha aquatica* | water mint | leaves, stalks, and inflorescences | leaves and stalks |
| *M. piperita* | peppermint | plant | leaves and stalks |
| *M. pulegium* | pennyroyal mint | plant | leaves and stalks |
| *Satureja montana* | winter savory | stalks and leaves | flowering tops |
| *Thymus vulgaris* | thyme | stalks and leaves | flowering tops |

## 2.1 Hydroethanolic extracts (HEEs)

The HEEs were dissolved in autoclaved deionized water for 20 min at an initial concentration of 10.0 mg/mL, in a flask covered with aluminum foil, using a magnetic stirrer, at room temperature and filtered through a Whatman filter paper No. 1, and, then, stirred for another 10 min. Working solutions were obtained by serial dilutions with autoclaved deionized water. The effect of the HEEs on *Meloidogyne incognita* J2 was tested at the concentrations of 5.0, 7.5, and 10.0 mg/mL. For the *Mentha pulegium* HEE the concentration of 2.5 mg/mL was tested instead of 7.5 mg/mL as no significant differences on the effect on J2 mortality for the concentrations of 5.0 and 10.0 mg/mL were observed (p>0.05).

Second-stage juveniles were obtained as described in 2.1, and only J2 collected within 72h were used. Twenty J2 were transferred individually to a well of a culture plate containing 2 mL of dissolved HEE, with a bristle. For each tested concentration, 4 repetitions were prepared, and autoclaved deionized water was used as control. The plates were kept in an incubator at 24 °C, and the number of mobile and immobile J2 was recorded at 1, 24, 48, 72 and 96 hours. Second-stage juveniles were considered immobile if they failed to react when probed with a bristle. The immobile J2 were, then, individually transferred to a glass block with autoclaved deionized water, and kept at room temperature, in the dark. After 1h, J2 that failed to react when probed again were

considered dead and those that showed movement were transferred into their respective well. Each bioassay was repeated 3 times.

## 2.2 Essential oils (EOs)

The EO solutions were prepared by dilution in autoclaved deionized water. Each work solution was prepared individually. The solutions were transferred to plastic tubes, covered with aluminum foil, and subjected to sonication with an ultrasonic probe, for 5 minutes, with an amplitude of 20%. When needed, the solutions were subjected again to sonication, in the same conditions. Afterwards, the tubes were kept on ice, in the dark. The effect of the EOs on *Meloidogyne incognita* J2 was tested at the concentrations of 500, 1000 and 2000 ppm. For the *C. citratus, S. montana* and *T. vulgaris* EOs the concentrations of 50, 100 and 250 ppm were also tested.

The bioassays were set up using the same methodology described in 2.2.1. The plates were sealed with parafilm in order to prevent the loss of volatile compounds. For each tested concentration, 4 repetitions were prepared, and autoclaved deionized water was used as control. Plates were incubated at 24 °C, and the number of mobile and immobile J2 was recorded at 1, 24, 48, 72 and 96 hours as described in 2.2.1. Each bioassay was repeated 3 times.

## 3. Effect of plant bioproducts on *M. incognita* second-stage juveniles (J2) infectivity

The effect of *C. citratus*, *Mentha aquatica* and *M. pulegium* HEEs and EOs on the infectivity of *Meloidogyne incognita* J2 was evaluated in micro-pot assays with tomato plants cv. Tiny Tim. The HEEs of *C. citratus* (8.5 mg/mL), *Mentha aquatica* (6.0 mg/mL) and *M. pulegium* (5.0 mg/mL), and EO solutions of *C. citratus* (250 ppm), *M. aquatica* and *M. pulegium* (2000 ppm) were prepared as described above.

The suspensions containing J2 were transferred to centrifuge tubes, and kept in a refrigerator at 4 °C for 1h, in order for J2 to precipitate. The tubes were centrifuged at 1500 rpm, for 5 min, at 5 °C and excess water was discarded. Five mL of the selected HEEs or EOs were added. The tubes were sealed and incubated, in the dark with agitation (≈ 100 rpm), at room temperature, for 48h. After incubation at 4 °C for 1h and centrifugation (1500 rpm, 5 min, 5 °C), the HEEs or EO solutions were discarded, and 5 mL of autoclaved deionized water were added to the J2 pellet at the bottom of each tube. Each tube was manually agitated for 2-3 min, and centrifuged as

described. This procedure was repeated 3 times to ensure no residues of HEEs or EOs were left in the J2 suspensions. The suspensions were transferred to glass beakers and the volume was adjusted to 10 mL.

Tomato seeds cv. Tiny Tim placed in plastic pots (40 cm$^3$) containing 25 cm$^3$ of autoclaved vermiculite moistened with nutrient solution (N:P:K, 6:3:6) were transferred to a growth chamber at 25 °C with a 12-hour photoperiod. After 10 days, the plants were uprooted and, after cleaning the roots to eliminate the vermiculite, transferred to pots containing 25 cm$^3$ of autoclaved soil mixture (turf:vermiculite:sand; 1:1:1). Then the plants were inoculated with 100 J2/pot obtained as described. Controls consisted of plants inoculated with J2 exposed to autoclaved sterile water and each treatment was replicated five times. The plants were kept in an air-conditioned room in the conditions described in 2.1.

Seven days after inoculation, the plants were uprooted and the roots were washed, weighed and stained with acid fuchsin (Byrd *et al.*, 1983). The numbers of nematodes inside the roots were counted using a stereomicroscope.

## 4. Effect of encapsulated *Cymbopogon citratus* essential oil, on *Meloidogyne incognita* second-stage juveniles (J2) mortality

The *Cymbopogon citratus* EO was selected for an assay against *M. incognita* J2, under an encapsulated formulation (ECCEO) in keratin microcapsules, kindly provided by Dr. Artur J. Ribeiro, of the Center for Biological Engineering (CEB) of the University of Minho, and kept in the dark, at 4 °C. The nematicidal activity of ECCEO on *M. incognita* J2 was tested *in vitro*, using the methodology described in 2.2.2. The bioassay consisted of ECCEO at the concentration of 250 ppm and *C. citratus* EO at the same concentration (prepared as described). For each treatment, 5 repetitions were prepared, and autoclaved deionized water and keratin as free protein, at the concentration present in the bioassay (100 ppm) were used as controls. The plates were sealed with parafilm, and incubated in the dark with agitation (≈ 90 rpm), at room temperature, for 48h. The number of mobile and immobile J2 was recorded as described in 2.2.1. In order to check the intake of the encapsulated EO by *M. incognita* J2, a new bioassay was performed with keratin microcapsules, and keratin, labelled with fluorescein isothiocyanate (FITC) (also provided by Dr. Artur J. Ribeiro). The bioassay was prepared using the same methodology previously referred in 2.4. After 48h, J2 exposed to ECCEO, and also free keratin stained with FITC, used as a control,

were mounted on glass slides in water, and photographed using a Olympus BX63 fluorescence microscope equipped with a DP74 camera. Imaging was processed with the green channel fluorescence, DIC and brightfield, using the Olympus cellSens software. Observations were made in at least three J2 specimens in each experimental condition.

## 5. Data analysis

Statistical analyses were performed using IBM SPSS Statistics for Windows, version 27.0 (Armonk, NY: IBM Corp).

Data on the effect of HEEs and EOs on the mortality of J2 of *Meloidogyne incognita* was converted into percentages of inhibitory effect, and corrected using the Abbott's formula (Abbott, 1925):

$$cm = (mt - mc) / (100 - mc) \times 100$$

where cm= corrected mortality (%), mt = mortality in treatment (%), mc = mortality in control (%). Datasets results of the biological replicates on number of dead J2 were normalized and compared using Generalized Linear Models (GLZM) assuming a normal distribution and identity link function (linear function), and the replicates were subjected to pairwise comparison, using the Least Significant Difference test (LSD) at probability levels of 5% ($p<0.05$). When a culture plate spilled, presented severe fungal contaminations, or the solution tested caused no effect in J2 as opposed to its biological replicates, as was the case with some EOs bioassays (due to improper dissolving), a new replicate was prepared. The experimental data was subjected to GLZM analysis, and when applicable, biological replicates were discarded.

Significant differences among the effect of different HEEs and EOs in J2 mortality (untransformed data) were investigated for each exposure time, through the use of Generalized Linear Mixed Models (GLMM), with a normal distribution, using the LSD test ($p<0.05$). In these models the plant species and the concentrations tested were used as fixed effects, and the bioassay replicates were used as random effects. Model outputs were corrected by the Satterthwaite approximation and using Robust Estimation. Significant differences among HEEs or EOs of different plant species were investigated with pairwise LSD test ($p<0.05$).

Data of the *in vitro* nematicidal activity of the HEEs and EOs (untransformed data) were subjected to Probit analysis for each exposure time, in order to estimate the $LC_{50}$ values for a maximum of 20 iterations at the different times of exposure.

Datasets results of the biological replicates on number of J2 per root gram, in infected roots were normalized and compared by GLZM analysis, using the same settings as described in J2 mortality analysis. Significant differences among the effect of HEEs and EOs in J2 infectivity (untransformed data) were investigated after 7 days, through the use of GLMM analysis, considering the plant species as fixed effects, and the bioassay replicates as random effects. Model outputs were corrected using the same settings as described in J2 mortality analysis.

Significant differences among the effect of ECCEO, *C. citratus* EO and keratin in J2 mortality were investigated after 48h, through the use of GLMM analysis, considering each sample as a fixed effect. Model outputs were corrected as described above.

# III. Results

# 1. Effect on *Meloidogyne incognita* second-stage juveniles (J2) mortality

The effect of different HEEs and EOs on the mortality of *M. incognita* J2 was evaluated *in vitro.*

## 1.1 Hydroethanolic extracts (HEEs)

### 1.1.1 *Cymbopogon citratus*

The *C. citratus* HEE caused no mortality of J2 in the first 24h, at any of the concentrations tested (Fig. 5). A clear increase in mortality was observed with the increase of exposure time. No significant differences were detected for the concentrations of 7.5 and 10.0 mg/ml at any time of exposure ($p>0.05$). After 96h, the *C. citratus* HEE affected 75.42, 86.25 and 88.75% of J2, at the concentration of 5.0, 7.5, and 10.0 mg/mL, respectively. No significant differences were observed among the different bioassays, after 72 and 96h of exposure ($p=0.19$, $p=0.57$, respectively).



**Figure 5.** Corrected percentage cumulative mortality of *Meloidogyne incognita* second-stage juveniles exposed to different concentrations of *Cymbopogon citratus* hydroethanolic extract at 5.0, 7.5 and 10.0 mg/mL. Values are means of combined data from three biological replicates with four repetitions for each treatment. Error bars represent the standard error.

### 1.1.2 *Mentha aquatica*

The *M. aquatica* HEE caused no mortality of J2 after 24h, at any of the concentrations tested (Fig. 6). After 48h, the highest nematicidal activity was observed at the concentration of 7.5 mg/mL, having affected 77.50% of J2 in comparison with 68.33% for the concentration of 10.0 mg/mL. After 72h, the HEE reached its maximum effect for the concentration of 5.0 mg/mL (64.58%). No significant differences were detected for the concentrations of 7.5 and 10.0 mg/mL, at any time of exposure ($p > 0.05$), nor among the different bioassays.



**Figure 6.** Corrected percentage cumulative mortality of *Meloidogyne incognita* second-stage juveniles, exposed to different concentrations (5.0; 7.5; 10.0 mg/mL) of *Mentha aquatica* hydroethanolic extract, after 24, 48, 72 and 96h. Values are means of combined data from three biological replicates with four repetitions for each treatment. Error bars represent the standard error.

### 1.1.3 *Mentha piperita*

The *M. piperita* HEE caused no mortality of J2 after 24h, at any of the concentrations tested (Fig. 7). A clear dose-response effect was observed afterwards. After 48h, the HEE affected 27.08 and 42.92% of J2 at the concentrations of 7.5 and 10.0 mg/mL, respectively. A clear increase in effect was observed over time, and after 96h of exposure, the HEE affected 29.17, 72.08 and 80.83% of J2, at the concentration of 5.0, 7.5 and 10.0 mg/mL, respectively. No significant differences were observed among the different bioassays, after 72h of exposure ($p = 0.11$).

**Figure 7.** Corrected percentage cumulative mortality of *Meloidogyne incognita* second-stage juveniles, exposed to different concentrations (5.0; 7.5; 10.0 mg/mL) of *Mentha piperita* hydroethanolic extract, after 24, 48, 72 and 96h. Values are means of combined data from three biological replicates with four repetitions for each treatment. Error bars represent the standard error.

### 1.1.4 *Mentha pulegium*

The *M. pulegium* HEE presented nematicidal activity after 24h (Fig. 8). A clear dose-response effect was not observed, since no significant differences were detected for the concentrations of 5.0 and 10.0 mg/mL, at any time of exposure (p>0.05). The HEE affected 9.17% of J2 at the concentration of 2.5 mg/mL, and 72.08 and 71.25% at the concentrations of 5.0 and 10.0 mg/mL, respectively. The mortality of J2 increased over time  with 15.83% of J2 affected at the concentration of 2.5 mg/mL, and 85.00 and 80.42% affected at the concentrations of 5.0 and 10.0 mg/mL, after 96h (p<0.05). No significant differences were observed among the different bioassays (p>0.05).
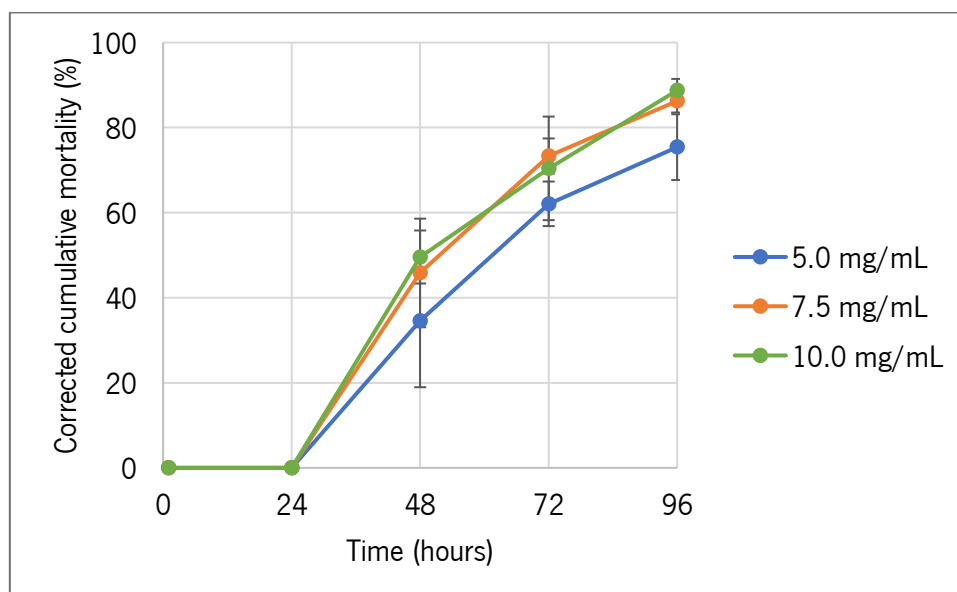
**Figure 8.** Corrected percentage cumulative mortality of *Meloidogyne incognita* second-stage juveniles, exposed to different concentrations (2.5; 5.0; 10.0 mg/mL) of *Mentha pulegium* hydroethanolic extract, after 24, 48, 72 and 96h. Values are means of combined data from three biological replicates with four repetitions for each treatment. Error bars represent the standard error.

### 1.1.5 *Satureja montana*

The *S. montana* HEE caused no mortality of J2 after 24h, at any of the concentrations tested (Fig. 9). A clear dose-response effect was observed afterwards. After 48h, the *S. montana* HEE affected 5.65 and 13.13% of J2 at the concentration of 7.5 and 10.0 mg/mL, respectively. A clear increase in mortality was observed with the increase of exposure time, for the concentration of 10.0 mg/mL, having affected 38.75% of J2, after 96h. The HEE caused no mortality of J2 at the concentration of 5.0 mg/mL, when compared with the control. No significant differences were observed among the different bioassays, after 72 and 96h (p=0.07, p=0.88, respectively).
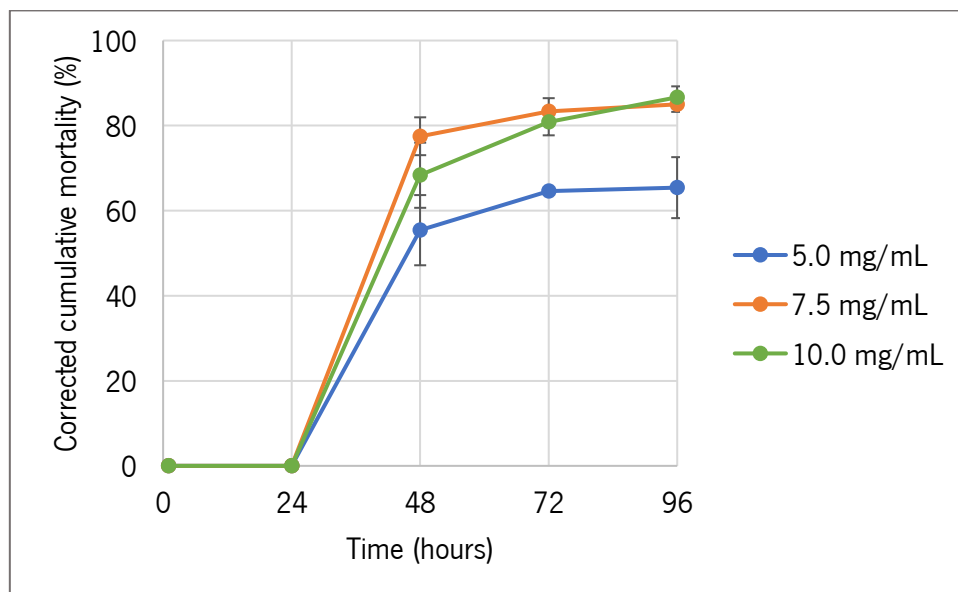
**Figure 9.** Corrected percentage cumulative mortality of *Meloidogyne incognita* second-stage juveniles, exposed to different concentrations (5.0; 7,5; 10.0 mg/mL) of *Satureja montana* hydroethanolic extract, after 24, 48, 72 and 96h. Values are means of combined data from three biological replicates with four repetitions for each treatment. Error bars represent the standard error.

### 1.1.6 *Thymus vulgaris*

The *T. vulgaris* HEE presented nematicidal activity after 24h of exposure (Fig. 10) causing 5.31, 6.56 and 9.06% mortality of J2 at the concentration of 5.0, 7.5 and 10.0 mg/mL, respectively. After 96h, the *T. vulgaris* HEE affected 86.88% of J2 at the concentration of 10.0 mg/mL. However, the results obtained in the prepared replicates were significantly different at every observation time (p<0.05), most likely due to contaminations, as this HEE was more prone to fungal contaminations.
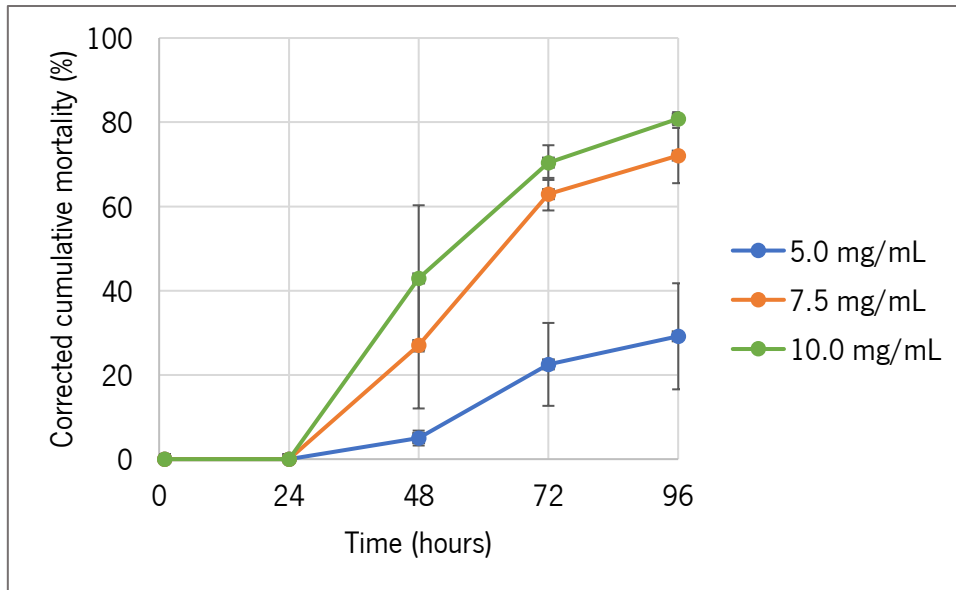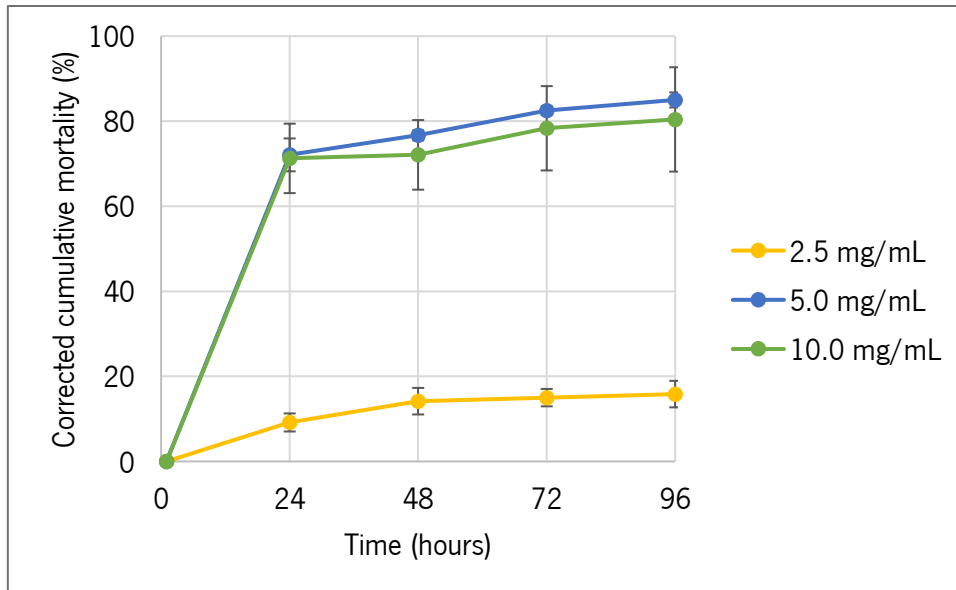
**Figure 10.** Corrected percentage cumulative mortality of *Meloidogyne incognita* second-stage juveniles, exposed to different concentrations (5.0; 7,5; 10.0 mg/mL) of *Thymus vulgaris* hydroethanolic extract, after 24, 48, 72 and 96h. Values are means of combined data from three biological replicates with four repetitions for each treatment. Error bars represent the standard error.

## 1.1.7 Effect of hydroethanolic extracts (HEEs) on *M. incognita* second-stage juveniles (J2)

After 24h, only the *Mentha pulegium* and *T. vulgaris* HEEs affected J2 of *Meloidogyne incognita* (Table 4). After 48h, the *Mentha aquatica* and *M. pulegium* HEEs presented the highest nematicidal activity, having affected an estimated mean of 44.55 and 46.75% of J2 (p=0.51). After 96h, the highest nematicidal activity was reported for *C. citratus*, *M. aquatica* and *M. pulegium* (p>0.05). The HEE of *S. montana* presented significantly lower nematicidal activity when compared to the other HEEs tested.

**Table 4.** Effect of hydroethanolic extracts on the mortality of second-stage juveniles (J2) of *Meloidogyne incognita.* Data are average % estimated by GLMM analysis.

| Plant Species | Dead J2[i] (%) | | | |
|---|---|---|---|---|
| | 24 | 48 | 72 | 96 |
| *Cymbopogon citratus* | NS | 28.44 (±3.53)b | 43.81 (±2.95)b | 52.94 (±2.92)a |
| *Mentha aquatica* | NS | 44.56 (±3.62)a | 48.84 (±3.05)ab | 50.42 (±3.01)ab |
| *M. piperita* | NS | 20.21 (±.3.58)c | 35.06 (±3.00)c | 40.27 (±2.97)c |
| *M. pulegium* | 42.71 (±2.75)a | 46.75 (±3.48)a | 51.72 (±3.00)a | 52.16 (±2.96)ab |
| *Satureja montana* | NS | 7.14 (±4.24)d | 9.76 (±3.73)d | 14.41 (±3.66)d |
| *Thymus vulgaris* | 4.55 (±2.84)b | 23.11 (±3.55)bc | 34.20 (±2.98)c | 45.86 (±3.21)bc |

[i] Percentages are combined data from three biological replicates experiments (± standard error)
NS - Non significant when compared with the control
Values followed by the same letter for each time of exposure are not significantly different according to LSD test (p>0.05)

## 1.1.8 Determination of lethal concentrations (HEEs)

Data of *in vitro* nematicidal activity of the different HEEs, after 48h, was subjected to Probit analysis. The lowest $LC_{50}$ values of 8.63, 5.98 and 5.32 mg/mL were obtained for the HEEs of *C. citratus, M. aquatica* and *M. pulegium.* The *M. piperita* and *S. montana* HEEs presented the weakest nematicidal activity, with $LC_{50}$ values of 9.73 and 14.44 mg/mL, respectively.

## 1.2 Essential oils (EOs)

### 1.2.1 *Cymbopogon citratus*

After 24h, the *C. citratus* EO affected 27.08, 82.92, 90.83 and 95.42% of J2 at the concentration of 250, 500, 1000 and 2000 ppm, respectively (Fig. 11). At the concentrations tested the EO reached its maximum effect after 24h, with the exception of the concentration of 250 ppm, which affected 36.67% of J2, after 96h. No significant differences were detected for the concentrations of 1000 and 2000 ppm at any time of exposure (p>0.05)
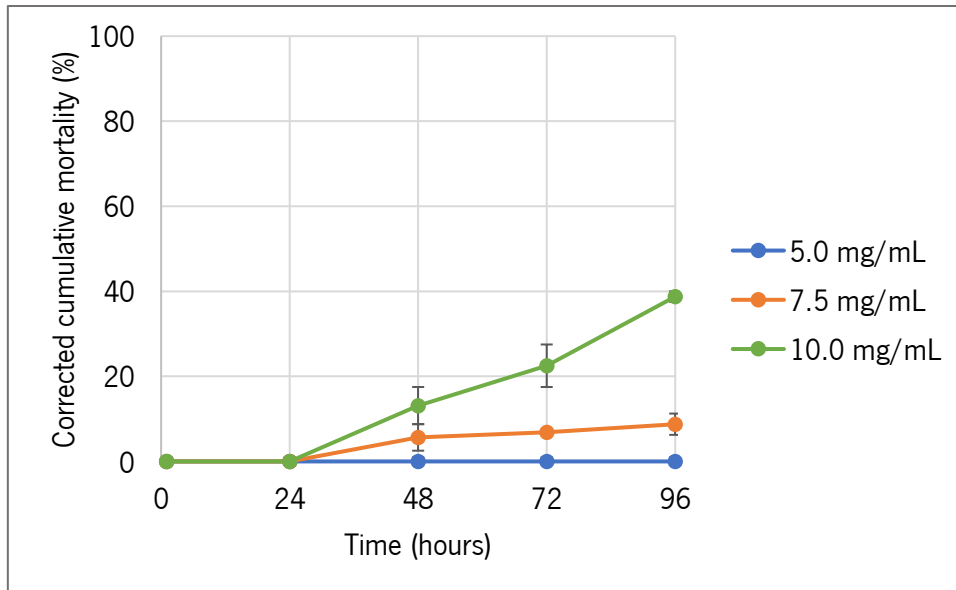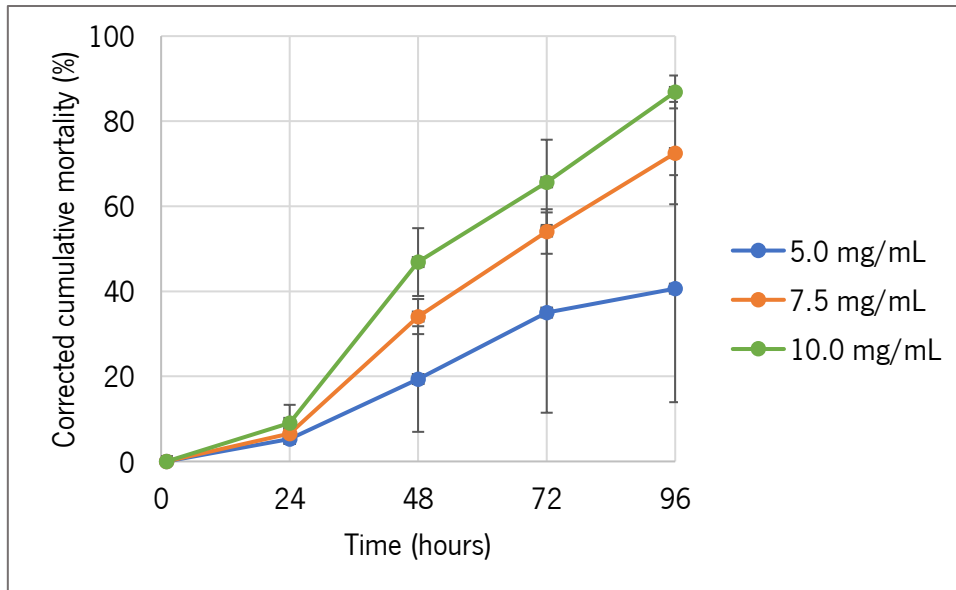
**Figure 11.** Corrected percentage cumulative mortality of *Meloidogyne incognita* second-stage juveniles, exposed to different concentrations (50; 100; 250; 500; 1000; 2000 ppm) of *Cymbopgon citratus* essential oil, after 24, 48, 72 and 96h. Values are means of combined data from three biological replicates with four repetitions for each treatment. Error bars represent the standard error.

### 1.2.2 *Mentha pulegium*

After 24h, the *M. pulegium* EO affected 2.92, and 28.33% of J2 of *M. incognita* at the concentration of 1000 and 2000 ppm (Fig. 12). The mortality of J2 exposed to *M. pulegium* EO increased over time, for the concentration of 1000 and 2000 ppm, but no nematicidal activity was observed at the concentration of 500 ppm, when compared with the results obtained in the control. After 96h, the concentration of 1000 and 2000 ppm affected 32.08 and 90% of J2, respectively.

**Figure 12.** Corrected percentage cumulative mortality of *Meloidogyne incognita* second-stage juveniles, exposed to different concentrations (500; 1000; 2000 ppm) of *Mentha pulegium* essential oil, after 24, 48, 72 and 96h. Values are means of combined data from three biological replicates with four repetitions for each treatment. Error bars represent the standard error.

### 1.2.3 *Satureja montana*

After 24h the *S. montana* EO affected 41.67% of J2 at the concentration of 500 ppm, and caused 100% of mortality at the concentrations of 1000 and 2000 ppm (Fig. 13). For the concentration of 500 ppm, an increase in effect was observed over time, until 72h of exposure, having affected 97,5% of J2. The EO had no effect in J2 of *M. incognita* after 96h of exposure to the concentration of 50, 100 and 250 ppm.
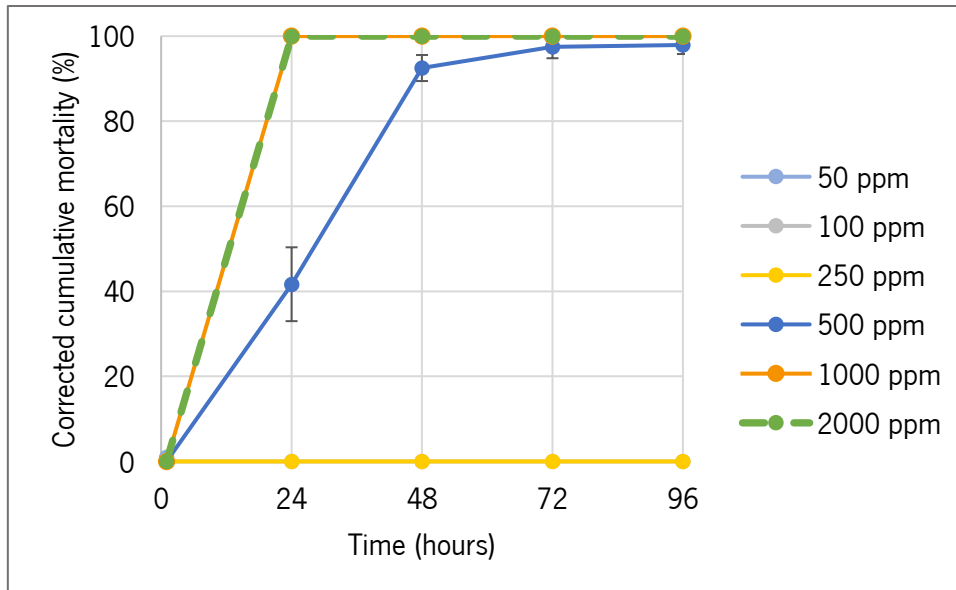
**Figure 13.** Corrected percentage cumulative mortality of *Meloidogyne incognita* second-stage juveniles, exposed to different concentrations (50; 100; 250; 500; 1000; 2000 ppm) of *Satureja montana* essential oil, after 24, 48, 72 and 96h. Values are means of combined data from three biological replicates with four repetitions for each treatment. Error bars represent the standard error.

### 1.2.4 *Thymus vulgaris*

After 24h, a dose-response effect was observed for the *T. vulgaris* EO, which affected 6.56, 64.69 and 96.25% of J2 at the concentrations of 500, 1000 and 2000 ppm (Fig. 14). After 48h the EO affected 100% of J2 at the concentrations of 1000 and 2000 ppm. The mortality of J2 exposed to the concentration of 500 ppm increased in a progressive manner over time, and after 96h, it affected 31.56% J2.
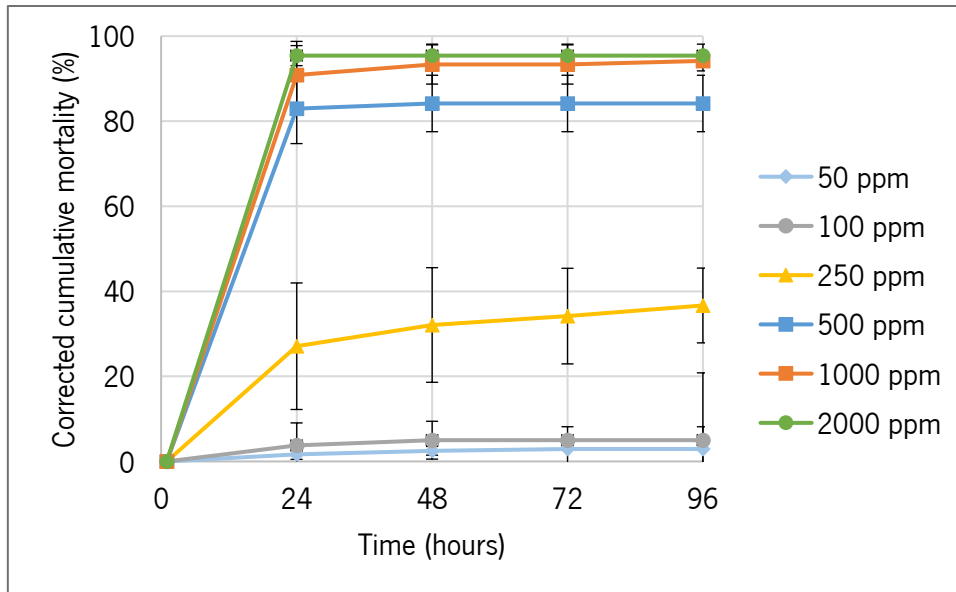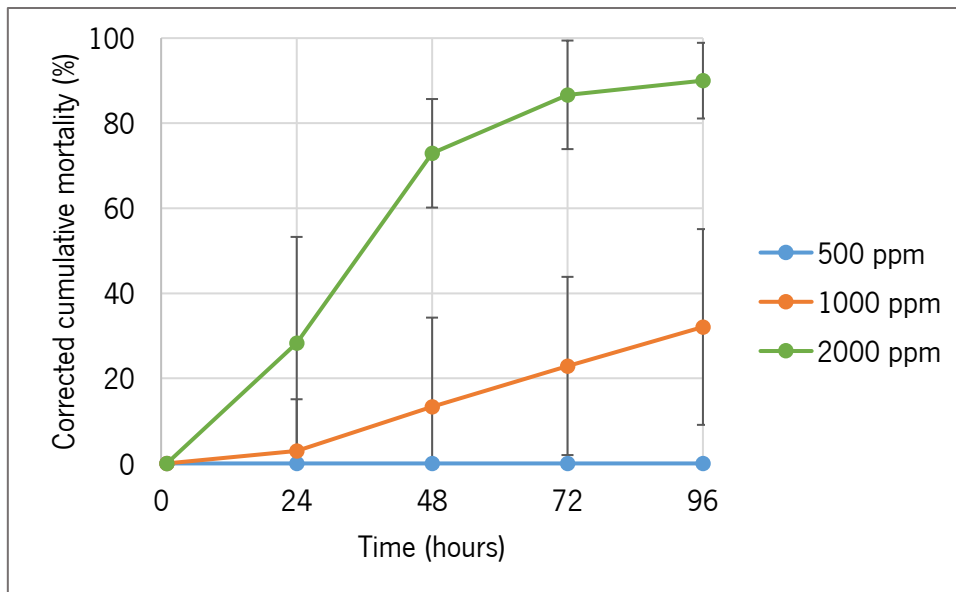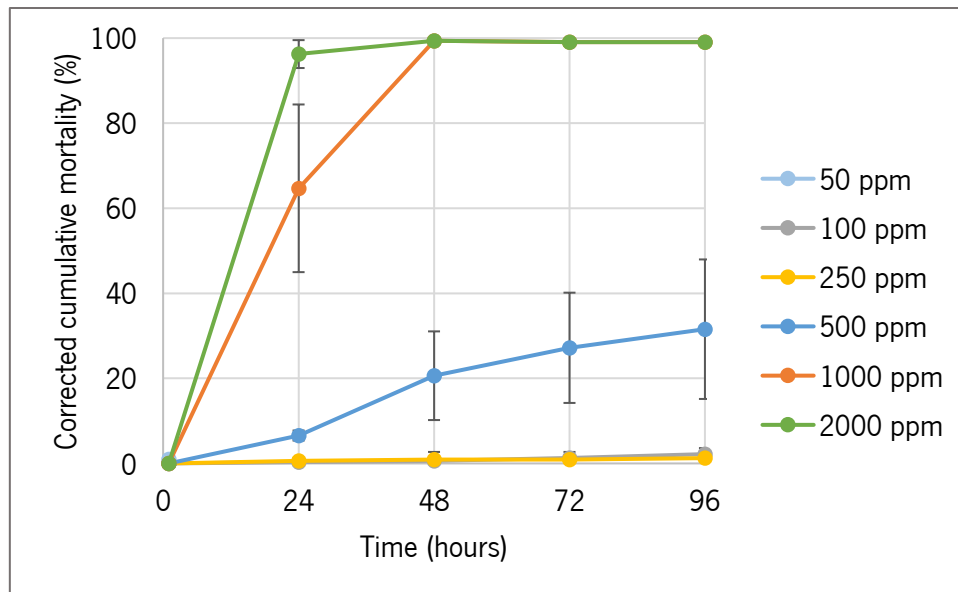
**Figure 14.** Corrected percentage cumulative mortality of *Meloidogyne incognita* second-stage juveniles, exposed to different concentrations (50; 100; 250; 500; 1000; 2000 ppm) of *Thymus vulgaris* essential oil, after 24, 48, 72 and 96h. Values are means of combined data from three biological replicates with four repetitions for each treatment. Error bars represent the standard error.

## 1.2.5 Effect of essential oils (EOs) on *M. incognita* second-stage juveniles (J2)

The results obtained by GLMM analysis of EOs are available on Table 5. After 24h the *C. citratus* EO presented the highest nematicidal activity, followed by *S. montana* EO, and affected an estimated mean of 43.59 and 33.88% of J2, respectively. The highest nematicidal activity observed after 48, 72 and 96h was also caused by the *C. citratus* EO, followed by *S. montana* and *T. vulgaris* EOs (p>0.05). The *Mentha aquatica* and *M. piperita* EOs had no effect in RKN J2, when compared with the control.

**Table 5.** Effect of essential oils on the mortality of second-stage juveniles (J2) of *Meloidogyne incognita*. Data are average % estimated by GLMM analysis.

| Plant Species | Dead J2[i] (%) | | | |
|---|---|---|---|---|
| | 24 | 48 | 72 | 96 |
| *Cymbopogon citratus* | 43.59 (±4.19)a | 44.52 (±5.02)a | 46.54 (±5.12)a | 47.75 (±5.00)a |
| *Mentha aquatica* | NS | NS | NS | NS |
| *M. piperita* | NS | NS | NS | NS |
| *M. pulegium* | NS | 7.33 (±5.58)c | 11.69 (±5.68)c | 14.40 (±5.58)c |
| *Satureja montana* | 33.88 (±4.22)b | 39.34(±5.06)ab | 39.75 (±5.16)b | 39.77 (±5.05)b |
| *Thymus vulgaris* | 24.56 (±4.12)c | 35.11 (±5.02)b | 36.24 (±5.12)b | 37.31 (±5.02)b |

[i] Percentages are average of combined data from three biological replicates experiments (± standard error)

NS - Non significant when compared with the control

Values followed by the same letter for each time of exposure are not significantly different according to LSD test (p>0.05)

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## 1.2.6 Determination of lethal concentrations (EOs)

The nematicidal activity of EOs after 48h could not be evaluated by Probit analysis, since after 20 iterations, a clear dose-response could not be observed for any EO. As such, the $LC_{50}$ values of EOs could not be properly calculated.

## 2. Effect on *Meloidogyne incognita* second-stage juveniles (J2) infectivity

The effect of different HEEs and EOs on the infectivity of J2 of *M. incognita* after 48h was evaluated in micro-pot assays as described in the previous section.

## 2.1 Hydroethanolic extracts (HEEs)

Datasets results of the biological replicates on number of J2 per root gram, in infected roots were normalized and compared using GLZM analysis, and the replicates were subjected to pairwise comparison, in order to check for significant differences. The third bioassay presented significant differences with the remaining bioassays ($p<0.05$), and as such, it was discarded.

Data regarding the effect of *C. citratus*, *Mentha aquatica* and *M. pulegium* HEEs in the infectivity of J2 of *Meloidogyne incognita* is available on Fig. 15. On average, after 7 days of infection there were 58, 68 and 54 J2 per root gram, in the plants infected with J2 exposed to $LC_{50}$ of HEEs of *C. citratus* (8.5 mg/mL), *Mentha aquatica* (6.0 mg/mL) and *M. pulegium* (5.0 mg/mL), which were significantly fewer than the average number of 174 J2 per root gram, observed in the control. Therefore, *C. citratus*, *M. aquatica* and *M. pulegium* HEEs, reduced the infectivity of J2 by 66.7, 60.9 and 69%.

**Figure 15.** Average number of second-stage juveniles (J2) of *Meloidogyne incognita* per root gram, after 7 days of infection. J2 were previously exposed to hydroethanolic extracts of *Cymbopogon citratus* (8.5 mg/mL), *Mentha aquatica* (6.0 mg/mL) or *M. pulegium* (5.0 mg/mL) for 48h. Error bars represent standard error. Columns with the same letter are not significantly different (p> 0.05) according to LSD test.

## 2.2 Essential oils (EOs)

Data regarding the effect of *C. citratus*, *M. aquatica* and *M. pulegium* EOs in the infectivity of J2 of *Meloidogyne incognita* is available on Fig. 16. On average, after 7 days of infection there were 24, 43 and 30 J2 per root gram, in the roots of plants infected with J2, exposed to selected concentrations of *C. citratus* (250 ppm), *Mentha aquatica* (2000 ppm) and *M. pulegium* (2000 ppm), which were significantly less than the average number of 192 J2 per root gram observed in the control. At the selected concentrations these EOs reduced the infectivity of J2 by 87.5, 77.8 and 84.4%, respectively. No significant differences were observed among the effect of the tested EOs, at the selected concentrations (p>0.05)
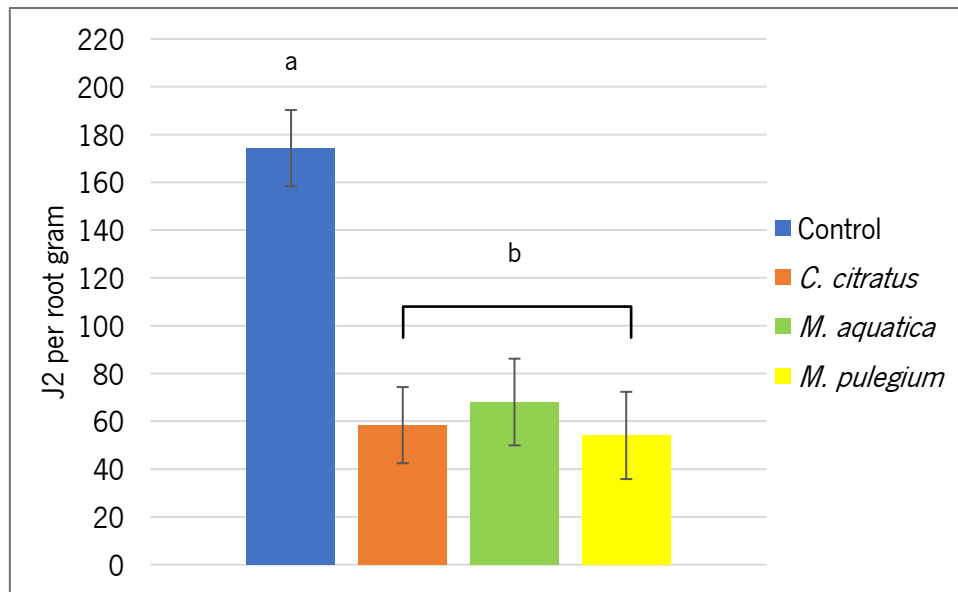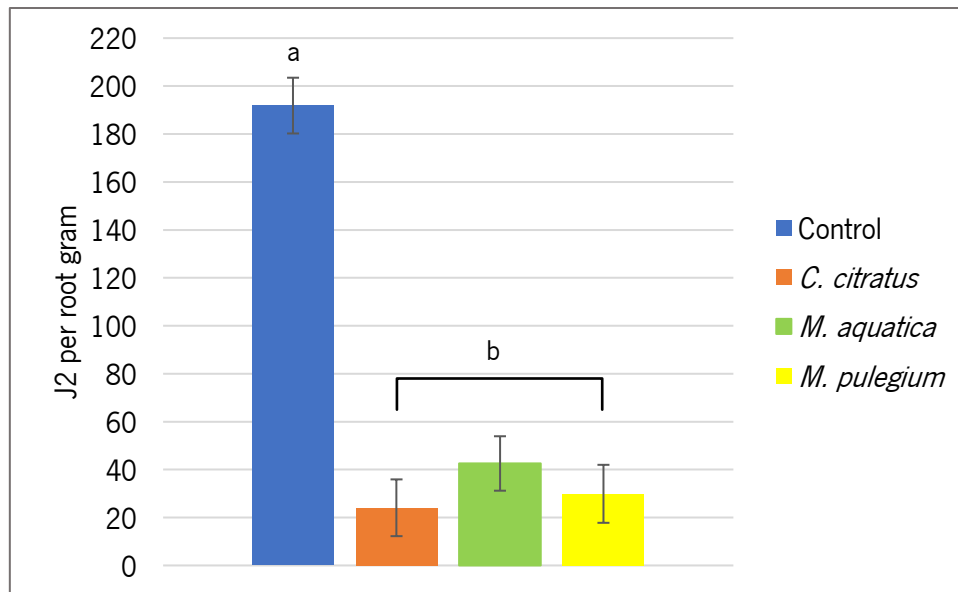
**Figure 16.** Average number of second-stage juveniles (J2) of *Meloidogyne incognita* per root gram, after 7 days of infection. J2 were previously exposed to selected concentrations of essential oils of *C. citratus*, (250 ppm), *Mentha aquatica* (2000 ppm) or *M. pulegium* (2000 ppm) for 48h. Error bars represent standard error. Columns with the same letter are not significantly different (p>0.05) according to LSD test.

## 3. Effect of encapsulated *Cymbopogon citratus* essential oil on *Meloidogyne incognita* second-stage juveniles (J2) mortality

The effect of encapsulated *Cymbopogon citratus* EO (ECCEO) on *M. incognita* J2 was tested. At the concentration of 250 ppm, the ECCEO caused an average mortality of 31% of J2, which was significantly higher than the 24% caused by *C. citratus* EO, at the same concentration (Table 6). No significant differences were observed among the results obtained with J2 exposed to keratin, when compared with the results obtained with J2 exposed to deionized water (p>0,05).

**Table 6.** Effect of *Cymbopogon citratus* essential oil (EO) and of encapsulated *C. citratus* EO (both at 250 ppm), and keratin (100 ppm) on the mortality of second-stage juveniles (J2) of *Meloidogyne incognita*, after 48h. Data are average % estimated by GLMM analysis.

| Sample | Dead J2[i] (%) |
|---|---|
| Deionized water | 6 (±1,90)c |
| Keratin | 11 (±5.53)c |
| *Cymbopogon citratus* | 24 (±3.55)b |
| Encapsulated *C. citratus* | 31 (±3.55)a |

[i] Percentages are average of five repetitions (± standard error) for each treatment.

Values followed by the same letter are not significantly different according to LSD test (p>0.05)

The intake of ECCEO by *M. incognita* J2 was observed by fluorescence microscopy. The J2 of *M. incognita* incubated with ECCEO showed fluorescence in the digestive system (Fig. 17A, B). A noticeable fluorescence could be observed in the intestine of J2 that died after incubation with ECCEO for 48h (Fig. 17A).
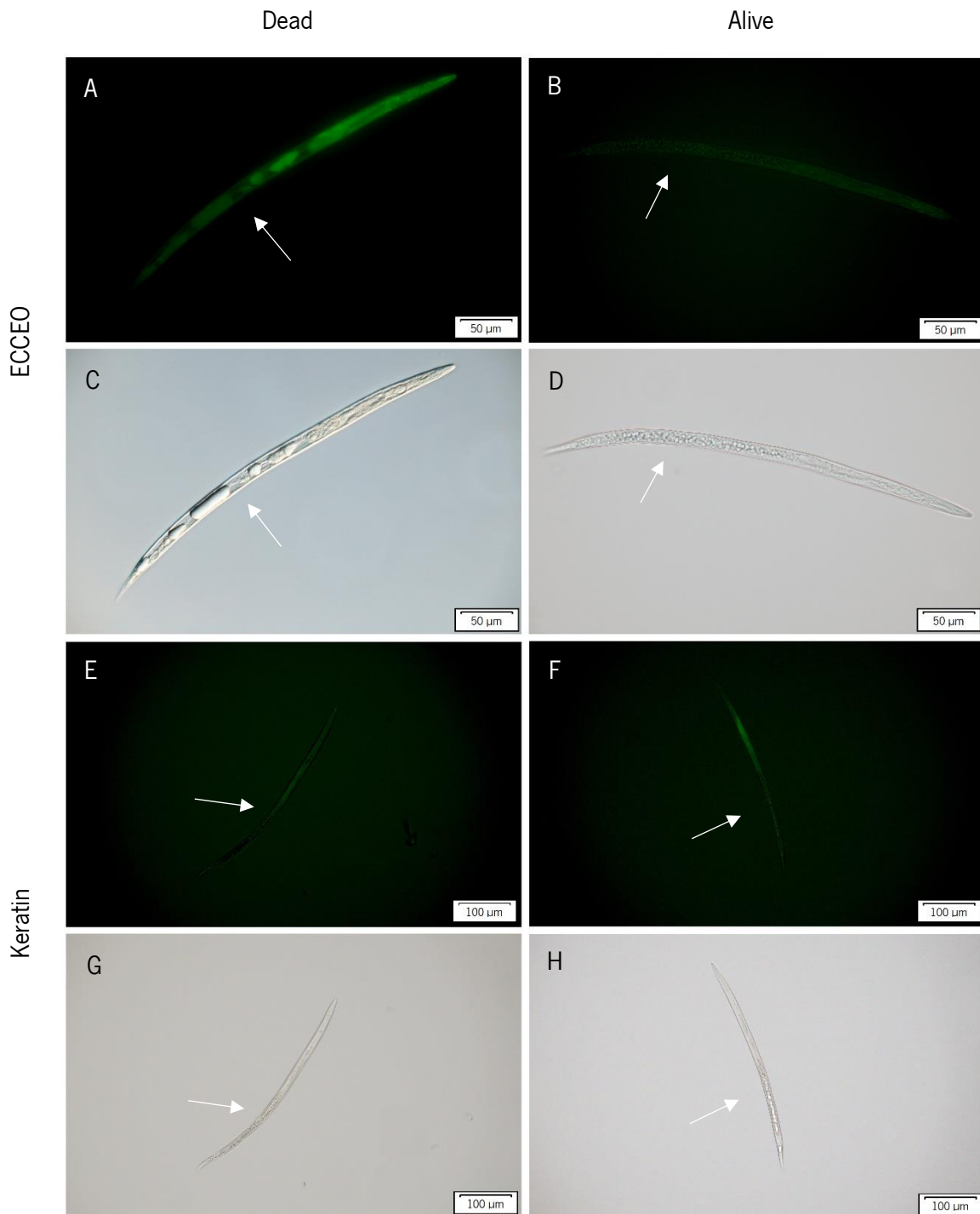


**Figure 17.** Fluorescence imaging of *Meloidogyne incognita* second-stage juveniles (J2), incubated for 48h, with *Cymbopogon citratus* essential oil encapsulated in keratin FITC labeled (A-D) and exposed to keratin labeled with FITC (E-H). Left images (A, C, E, G), dead J2; Right images (B, D, F, H), alive J2. C - imaged with DIC; D, G, H – brightfield images. Arrows indicate the median intestine region.

The *M. incognita* J2 that died after incubation also showed some morphological changes in the intestine region (Fig. 17A, C). In the J2 that were alive after incubation, these kind of morphological changes were not observed (Fig. 17D), or in J2 exposed just to keratin (Fig. 17H).

Labeled keratin was used as a control, and the fluorescence of exposed J2 could also be observed along their digestive system (Fig. 17E, F). No morphological changes were observed in the intestine of J2 that were dead after incubation with this protein, for 48h (Fig. 17G, H).

# IV. Discussion

The search for green pesticides from natural sources is important for the development of new botanical pesticides, and control of RKN through the use of plant extracts and essential oil (EO) based formulations can be an economically rewarding for agricultural systems, due to the lack of syntethic nematicides options, and since plant extracts and EOs can be a source of cheap and effective nematicides (Corbani *et al.*, 2013; Watson and Desaeger, 2019).

In this study the hydroethanolic extracts (HEEs) and EOs of *Cymbopogon citratus*, *Mentha aquatica*, *M. piperita*, *M. pulegium*, *Satureja montana* and *Thymus vulgaris* effect on the mortality of J2 of *Meloidogyne incognita* was evaluated. Only the *Mentha pulegium* and *T. vulgaris* HEEs affected J2 after 24h. The *M. pulegium* and *M. aquatica* HEEs were the most effective (p>0.05), followed by the *C. citratus* HEE, with LC$_{50}$ values of 5.32, 5.98 and 8.63 mg/mL, respectively (Table 4). None of the tested HEE presented mortality values of over 90% after 96h, at the maximum concentration tested of 10.0 mg/mL. The HEEs of *C. citratus* and *M. aquatica* seem to have reached their maximum effect at the concentration of 7.5 mg/mL, and *M. pulegium* at the concentration of 5.0 mg/mL, since no differences in results were observed at higher concentrations (p>0.05). Every HEE presented some level of fungal contaminations over time, and it seemed to be proportional with the increase in concentration, with *T. vulgaris* HEE being the most prone to contamination throughout the bioassays.

The *C. citratus, M. aquatica,* and *M. pulegium* HEEs were tested in regards to their effect in the infectivity of J2. After 7 days of infection, the number of J2 per root gram was reduced by 66.7, 60.9 and 69% (Fig. 15).

There is little information regarding the nematicidal activity of HEEs of the plant species used in this study, as only the methanolic extracts of *M. piperita* and *M. pulegium* have been previously tested in regards to their effect on the mobility of J2 of *Meloidogyne incognita*, and the maximum concentration tested of 1000 ppm was not enough to affect 50% of exposed J2. (Caboni *et al.*, 2013).

Of the tested EOs, *Mentha aquatica* and *M. piperita* EOs had no effect on the mortality of J2. The remaining EOs had an effect on J2 after 24h, and the most effective EOs were of *C. citratus*, followed by *S. montana* and *T. vulgaris* (Table 5). These three EOs affected approximately 100% of J2 after 24 of exposure to the maximum concentration tested of 2000 ppm (Figs. 11, 13 and 14). After 48h, these EOs also affected more than 90% of J2 at the concentration of 1000 ppm, although only the *C. citratus* EO had effect on J2 at concentrations lower than 500 ppm (Fig. 11). As opposed

to what was observed in HEEs bioassays, no sort of contamination was observed throughout the EOs bioassays, most likely due to the antimicrobial nature of EOs (Ibrahim *et al.*, 2006).

The equivalent EOs of the HEEs tested on the infectivity of J2 were also tested in regards to their effect on infectivity. Since no dose-response effect was observed for the EOs after 48h of exposure, concentrations were selected based on the mortality results (Figs. 11 and 12). Therefore, of the EOs tested on the effect on the infectivity of J2, *C. citratus* EO was the most effective, as it required a lower concentration, followed by the *M. pulegium* and *M. aquatica* EOs. Despite not having an effect in the mortality of J2*, M. aquatica* EO was able to affect the infectivity of J2, at the concentration of 2000 ppm. No significant differences in effect was observed among the results obtained with the selected concentrations of EOs (p>0.05). Significant differences were observed however, among the results obtained in biological replicates (p<0.05), which may have been caused by improper dissolution of EOs.

*Cymbopogon citratus* EO has been tested by several authors in regards to its effect on RKN J2, and in reports with similar conditions, such as exposure time and concentrations, the EOs presented superior results to the obtained in this study (Gupta *et al.*, 2011; Barros *et al.*, 2020). This may be due to the use of surfactants such as Tween 80, which may have an influence in J2 health. Results obtained by different authors with *M. piperita* EO are variable. Some works report some noticeable effects in regards to mobility of *Meloidogyne javanica* (Oka *et al.*, 2000; Ntalli *et al.*, 2010) and mortality of J2 of *M. incognita* and *M. javanica* (Pandey *et al.*, 2000; Amora *et al.*, 2017), while other studies report no nematicidal effect in J2 of these two species (Caboni *et al.*, 2013; Andrés *et al.*, 2012). *Mentha pulegium* EO has been previously tested and caused no effect on J2 of *Meloidogyne incognita*, after 72h to exposure (1500 ppm) (Caboni *et al.*, 2013), which differs with the results obtained with the provided EO, as it affected 22.9 and 86.7% of J2, after 72h of exposure to the concentrations of 1000, and 2000 ppm. In this work, *S. montana* EO was effective against J2 of *M. incognita*, since 100% mortality of J2 was induced, at the concentration of 1000 ppm, after 24h, and the nematicidal activity of this EO has also been previously reported in another work, after inducing the mortality of 100% of *M. javanica* J2, at the same concentration, after 12h (Andrés *et al.*, 2012). In regards to *T. vulgaris* EO, in a similar study, it proved effective as it induced 99.4% mortality at the concentration of 2500 ppm, after 24h (Amora *et al.*, 2017), which was also observed in the results obtained in this study, since the EO caused the mortality of 96.3% of J2 after 24h, at the concentration of 2000 ppm. A lower effect than the observed in this work has also been reported, after the EO caused the mortality of approximately 70% of J2, after

48h of exposure to the concentration of 1000 ppm (Barros *et al.*, 2019), and a higher effect was reported (100% mortality of J2 at the concentration of 1000 ppm, after 12h exposure (Andrés *et al.*, 2012). The inconsistency in results observed among the EOs bioassays, is most likely due to improper dissolution of EOs, since, with the small solution volumes prepared, a slight fraction of undiluted EO is enough to highly influence the final concentration of the work solutions, and since no surfactants were used, when preparing the EO solutions.

Overall, the tested EOs, were more biologically active against *M. incognita* J2, since some were able to affect 100% of J2 at certain concentrations after 24h, as oppose to the tested HEEs, that were not able to affect over 90% of J2, at the maximum concentration tested of 10.0 mg/mL, even after 96h. The *S. montana* HEE presented the lowest nematicidal activity of the tested HEEs, while the EO was one of the most effective, despite not having any effect at concentrations lower than 500 ppm. *C. citratus* was the plant that presented the highest nematicidal potential of the tested species, and was selected for a bioassay under an encapsulated formulation.

The effect of keratin encapsulated *Cymbopogon citratus* EO (ECCEO) on the mortality of J2 was investigated. After 48h, the ECCEO affected a superior number of J2, than the *C. citratus* EO in its free formulation (p>0.05). Free keratin was used as a control, and had no significant effect in the mortality of J2, when compared with the results obtained with J2 exposed to deionized water (p<0.05) (Table 6). Images obtained by fluorescence microscopy show fluorescence throughout the digestive system of J2 incubated with ECCEO and with keratin (Fig. 17). Some fluorescence was found in the anterior region of the J2 in both types of exposure, either to the ECCEO, or to the protein, which may indicate that some fluorescein could have diffuse in this region, since no fluorescence was observed in J2 that were exposed to deionized water. However, a noticeable fluorescence was observed in J2 that died after incubation with ECCEO (Fig. 17A), when compared with J2 that died after exposure to only keratin (Fig. 17E), which may indicate that *C. citratus* EO (inside the keratin nanoparticles) was ingested. The *M. incognita* J2 that died after incubation with ECCEO showed some morphological changes in the intestine middle region (Fig. 17C). The ECCEO had no apparent effect in J2 that were alive after incubation, since no differences in fluorescence or morphology could be observed (Fig. 17B, D), when compared with J2 incubated with only keratin (Fig. 17F, H). These results seem promising, since the encapsulation is a potential solution to the persistence problems of botanical extracts and EOs, and to the solubility problems of EOs (Devi et. al 2019). More biological replicates are required in order to properly evaluate and confirm the effect of ECCEO on *M. incognita* J2.

The present study on plant extracts and EOs supports their biopesticidal nature. The results obtained seem to indicate that the HEEs of *C. citratus*, *Mentha aquatica* and *M. pulegium*, and EOs of *C. citratus*, *M. pulegium*, *Satureja montana* and *Thymus vulgaris* show potential for the development of new nematicidal formulations, that are sustainable to the environment and human health, and can be incorporated in IPM programs. However, considering that the chemical composition of plant extracts and EOs varies upon various factors such as agronomical factors, plant age, and technical procedures, such as plant material drying and EO extraction, a standardization of these parameters is needed in order to obtain safe and effective commercial formulations (Andrés *et al.*, 2012; Ashoka *et al.*, 2017). Additional trials are needed to further validate their potential application in the management of RKN.

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