ACTIVITY OF ESSENTIAL OILS AND COUMARINS AGAINST THE NEMATODES *CAENORHABDITIS ELEGANS* AND *ANISAKIS SIMPLEX*

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Tiivistelmä/Referat – Abstract

Anisakiasis is a parasitic disease caused by larval nematodes of the genus *Anisakis*. Humans become infected by consuming contaminated raw or undercooked seafood products. Most human infections are caused by *Anisakis simplex* (*A. simplex*) complex. Currently there is no effective drug for this global emerging disease. Novel active compounds against the nematode are needed for drug development purposes. The research with *A. simplex* requires the isolation of the larvae from fish, which is time-consuming, unecological and uneconomical. Thus, the utilization of the model nematode *Caenorhabditis elegans* (*C. elegans*) in the research of *A. simplex* is considered in this study.

Activities of Tea tree, Java citronella and Ho wood essential oils against *C. elegans* were studied. Aim of the assays was to examine whether *C. elegans* could be used as a model for *A. simplex*. Observed effects on *C. elegans* were compared to the previously reported effects on *A. simplex*. Activity of Tea tree and Java citronella essential oils against *A. simplex* was also examined to confirm previously reported activity. In addition, activity of six coumarins against *A. simplex* was investigated. The aim of the assays was to discover novel active compounds against the pathogenic nematode. Four coumarins were tested against *C. elegans* to examine possible comparable effects. Toxicity studies were performed in aquatic medium in a 6 well plate format (*A. simplex*) and in a 96 well plate format or in 1.5 mL Eppendorf tubes (*C. elegans*).

Tea tree essential oil showed dose dependent activity against *C. elegans*, producing 100% mortality with the concentration 20 µL/mL after 24 hours exposure. Compared to *A. simplex*, two to three times higher doses were required to produce same degree of mortality in *C. elegans*. By contrast, Java citronella and Ho wood essential oils showed no significant activity against *C. elegans*. The activity of Tea tree and Java citronella essential oils against *A. simplex* was confirmed. The tested coumarins displayed no significant activity against the nematodes. Due to the contradictory results, further investigation about the suitability of *C. elegans* as a model for *A. simplex* is needed. Differences between the effective concentrations are probably caused by the differences in the biology of the nematodes, which result from the phylogenetic distance. Based on current results, the tested coumarins were excluded as potential antinematodal compounds against *A. simplex*, due to the lack of any significant activity on this model.

Avainsanat – Nyckelord – Keywords

Anisakis simplex, *Caenorhabditis elegans*, coumarins, essential oils, toxicity studies, model nematode, anisakiasis

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Tiivistelmä/Referat – Abstract

Anisakiaasi on *Anisakis*-sukuun kuuluvien sukkulamatojen toukkien aiheuttama loistauti. Ihmiset saavat tartunnan syömällä kontaminoituneita raakoja tai huonosti kypsennettyjä merenelävіä. Useimmat ihmisen infektiot ovat *Anisakis simplex* (*A. simplex*) -kompleksin aiheuttamia. Tällä hetkellä tähän maailmanlaajuiseen lisääntyvään tautiin ei ole tehokasta lääkettä. Tätä sukkulamatoa vastaan tarvitaan uusia aktiivisia yhdisteitä lääkekehitystarkoituksiin. *A. simplex* -tutkimus edellyttää toukkien eristämistä kaloista, mikä on aikaa vievää, epäekologista ja epätaloudellista. Täten tässä työssä tarkastellaan *Caenorhabditis elegans* (*C. elegans*) -sukkulamatomallin hyödyntämistä *A. simplex* -tutkimuksessa.

Teepuun, Java sitronellan ja Ho-puun eteeristen öljyjen aktiivisuuksia tutkittiin *C. elegans* -matoa vastaan. Kokeiden tarkoituksena oli selvittää, voitaisiinko *C. elegans* -matoa käyttää *A. simplex* -madon mallina. *C. elegans* -madossa havaittuja vaikutuksia verrattiin aiemmin raportoituihin vaikutuksiin *A. simplex* -madossa. Myös Teepuun ja Java sitronellan eteeristen öljyjen aktiivisuutta *A. simplex* -matoa vastaan tutkittiin aiemmin raportoitujen vaikutusten varmistamiseksi. Lisäksi kuuden kumariinin aktiivisuutta *A. simplex* -matoa vastaan tutkittiin. Kokeiden tarkoituksena oli löytää uusia aktiivisia yhdisteitä patogeenistä sukkulamatoa vastaan. Neljää kumariinia testattiin *C. elegans* -matoa vastaan mahdollisten vertailukelpoisten vaikutusten tarkastelemiseksi. Toksisuustutkimukset suoritettiin vesiviljelyaineessa 6-kuoppalevyillä (*A. simplex*) ja 96-kuoppalevyillä tai 1,5 ml:n Eppendorf-putkissa (*C. elegans*).

Teepuun eteerinen öljy osoitti annoksesta riippuvaa aktiivisuutta *C. elegans* -matoa vastaan, aiheuttaen 100 % kuolleisuuden pitoisuudella 20 µl/ml 24 tunnin altistuksen jälkeen. Verrattuna *A. simplex* -matoihin, kahdesta kolmeen kertaa suurempia annoksia tarvittiin tuottamaan samanasteinen kuolleisuus *C. elegans* -madoissa. Sitä vastoin Java sitronellan ja Ho-puun eteeriset öljyt eivät osoittaneet merkittävää aktiivisuutta *C. elegans* -matoa vastaan. Teepuun ja Java sitronellan eteeristen öljyjen aktiivisuus *A. simplex* -matoa vastaan vahvistettiin. Testatut kumariinit eivät osoittaneet merkittävää aktiivisuutta sukkulamatoja vastaan. Ristiriitaisista tuloksista johtuen lisätutkimuksia tarvitaan *C. elegans* -madon sopivuudesta *A. simplex* -madon malliksi. Erot vaikuttavien pitoisuuksien välillä aiheutuvat todennäköisesti sukkulamatojen biologisista eroista, jotka johtuvat fylogeneettisestä etäisyydestä. Tämänhetkisten tulosten perusteella testatut kumariinit hylättiin potentiaalisina *A. simplex* -sukkulamatoon tehoavina yhdisteinä, koska merkittävää aktiivisuutta ei havaittu tässä mallissa.

Avainsanat – Nyckelord – Keywords

Anisakis simplex, *Caenorhabditis elegans*, kumariinit, eteeriset öljyt, toksisuustutkimukset, sukkulamatomalli, anisakiaasi

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Kristiina Tukiainen

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LIST OF ABBREVIATIONS

A. besseyi	*Aphelenchoides besseyi*
A. simplex	*Anisakis simplex*
B. mucronatus	*Bursaphelenchus mucronatus*
B. xylophilus	*Bursaphelenchus xylophilus*
C. elegans	*Caenorhabditis elegans*
C7	Coumarin 7
C30	Coumarin 30
C102	Coumarin 102
C153	Coumarin 153
D. destructor	*Ditylenchus destructor*
DMSO	dimethyl sulfoxide
E. coli	*Escherichia coli*
EO	essential oil
FDA	U.S. Food and Drug Administration
IgA	immunoglobulin A
IgE	immunoglobulin E
IgG	immunoglobulin G
LB Broth	Luria Bertani Broth
LC_{50}	median lethal concentration
LD_{50}	median lethal dose
L1	first stage
L2	second stage
L3	third stage
L4	fourth stage
M. incognita	*Meloidogyne incognita*
MW	molecular weight

NGM	nematode growth medium
rpm	revolutions per minute
v/v	volume/volume

1 INTRODUCTION

Anisakiasis is a parasitic disease caused by nematodes of the genus *Anisakis* (Baptista-Fernandes et al. 2017). Humans become infected by consuming seafood products, which are contaminated by third stage (L3) larvae of the nematodes. Most human infections are caused by species of *Anisakis simplex* (*A. simplex*) complex (Beaudry 2012). Anisakiasis is a worldwide disease, mainly diagnosed in countries where raw or undercooked seafood products are an important part of the traditional food culture (Pellegrini et al. 2004; Baptista-Fernandes et al. 2017). The disease is endemic in Japan, but several cases have been diagnosed in coastal areas of Europe, such as Mediterranean countries. Currently, there is no proven pharmacological treatment for anisakiasis and effort has been made to discover active compounds against *A. simplex*. However, research with *A. simplex* involves the isolation of the larvae from fish, since the parasite cannot maintain its life cycle under laboratory conditions (Partridge et al. 2018). This method is ecologically and economically unsustainable causing the waste of food. The challenging maintenance of parasitic nematodes in artificial conditions has evoked the interest in more useful model system (Holden-Dye and Walker 2014). Thus, *Caenorhabditis elegans* (*C. elegans*), a free-living nematode which can be easily cultivated in the laboratory has been exploited as a model organism for parasitic nematodes (Blaxter 2011; Holden-Dye and Walker 2014). To our best knowledge, this model nematode has not been used in the research of *A. simplex*.

This thesis focused on exploring the first steps in the discovery of drugs active against *A. simplex* using natural sources (essential oils and coumarins). In drug discovery programs natural products have been utilized as a source of new bioactive compounds for decades (Newman and Cragg 2016). Plant secondary metabolites, such as essential oils and coumarins, comprise a diverse class of compounds with varied biological properties. Several essential oils have shown activity against *A. simplex*, including Tea tree essential oil (EO) with a dose dependent activity (Gómez-Rincón et al. 2014; Valero et al. 2015). In the experimental part of this thesis, activity of Tea tree EO

against *C. elegans* was examined. The aim of these assays was to determine whether *C. elegans* could be used as a model organism in the research of *A. simplex*. This could be possible if Tea tree EO has a comparable effect on *C. elegans* (Kearn et al. 2014). In addition, activity of Java citronella and Ho wood essential oils against *C. elegans* was investigated, since there are previous findings about their lethal effect on *A. simplex* (Valero et al. 2015; Gómez-Rincón C, personal notice 25th April 2016). Several coumarin compounds have shown activity against plant parasitic and free-living nematodes (Mahajan et al. 1992; Takaishi et al. 2008; Wang et al. 2008; Liu et al. 2011; Pan et al. 2016). Therefore, coumarins were chosen for the toxicity studies against *A. simplex*. Activity of six coumarins (Coumarin, Coumarin 7 (C7), Coumarin 30 (C30), Coumarin 102 (C102), Coumarin 153 (C153) and *m*-Coumaric acid) against *A. simplex* and *C. elegans* was examined. The main purpose of these assays was to discover novel compounds against the pathogenic nematode *A. simplex* for further drug development steps. The assays performed with *C. elegans* were aimed at examining the possible comparable effects of active compounds.

2 LITERATURE REVIEW

This literature review comprises of four parts. The first chapter gives an overview of anisakiasis, which is the medical disorder of focus in this thesis. In the second chapter, the general aspects of the target organism (*A. simplex*) and the model species used for the study of *A. simplex* (*C. elegans*) are introduced. In the final two chapters, the natural products relevant to this study (i.e. essential oils and coumarins) are presented.

2.1 Anisakiasis

Anisakiasis is a foodborne zoonosis caused by the L3 larvae of the nematodes belonging to the genus *Anisakis* (Shimamura et al. 2016; Baptista-Fernandes et al. 2017). The majority of human infections are caused by *A. simplex sensu lato* (s.l.), which is a complex of three sibling species: *A. simplex sensu stricto* (s.s.), *Anisakis pegreffii* (*A. pegreffii*) and *A. simplex* C (Klimpel and Palm 2011; Beaudry 2012; Buchmann and Mehrdana 2016). These morphologically similar sibling species can be differentiated only by genetic analysis (Klimpel and Palm 2011; Beaudry 2012). In this study, *A. simplex* refers to the species complex.

2.1.1 Epidemiology

Anisakis species (*Anisakis* spp.) are widely distributed throughout the world's oceans (Acha and Szyfres 2003; Kuhn et al. 2011). The sibling species of *A. simplex* complex are mainly found in the Atlantic Ocean, the East and West Pacific and the Mediterranean Sea (Kuhn et al. 2011). Several fish species, such as herring, mackerel, codfish, anchovy and wild salmon, or squid act as transport hosts for *A. simplex* L3 larvae (Pellegrini et al. 2004). Dishes containing raw or undercooked (e.g. marinated,

pickled, salted or smoked) seafood products pose a risk of infection (Pellegrini et al. 2004; Baptista-Fernandes et al. 2017). Common sources of infection include Japanese sushi and sashimi, Hawaiian lomi-lomi, Latin American ceviche, Dutch or German herring, Nordic gravlax, Spanish boquerones en vinagre and Italian alici marinate. Epidemiologically speaking, anisakiasis is a worldwide disease recognized mainly in countries where traditional cuisine contains raw or undercooked seafood products. The first diagnosed human infection was reported in the 1960s from the Netherlands (Audicana and Kennedy 2008; Beaudry 2012). To date, the reported number of anisakiasis cases globally exceeds 20,000 (Baptista-Fernandes et al. 2017). The vast majority (over 90%) of cases are from Japan, where approximately 2,000 cases are reported each year (Pellegrini et al. 2004; Baptista-Fernandes et al. 2017). The rest of the cases have been reported in all inhabited continents. The countries include Korea, Taiwan, the Netherlands, Croatia, the United Kingdom, Norway, France, Spain, Italy, Portugal, Canada, the United States, South Africa and Australia among others (Lucas et al. 1985; Bouree et al. 1995; Eskesen et al. 2001; Pellegrini et al. 2004; Nieuwenhuizen et al. 2006; Bucci et al. 2013; Mladineo et al. 2014; Li et al. 2015; Shimamura et al. 2016; Baptista-Fernandes et al. 2017; Zanelli et al. 2017).

2.1.2 Clinical features

The degree of anisakiasis depends on the larvae location, varying from asymptomatic disease to severe complications. In noninvasive luminal anisakiasis, larvae remain in the digestive tract lumen without penetrating the tissues (Sakanari and McKerrow 1989; Baptista-Fernandes et al. 2017). The infection is usually asymptomatic and larvae may be discovered accidentally in the sputum, vomit or stool of the patient (Sakanari and McKerrow 1989; Acha and Szyfres 2003; Baptista-Fernandes et al. 2017). Invasive anisakiasis is more common, appearing in gastric, intestinal or ectopic form (Sakanari and McKerrow 1989; Hochberg and Hamer 2010; Baptista-Fernandes et al. 2017). In gastric and intestinal anisakiasis, larvae penetrate the gastrointestinal wall (mucosa and submucosa), causing direct tissue damage with acute inflammation (Pellegrini et al.

2004; Mineta et al. 2006; Audicana and Kennedy 2008). After the ingestion of contaminated food, symptoms develop within 1–12 hours in acute gastric form and within 5–7 days in acute intestinal form (Hochberg and Hamer 2010; Baptista-Fernandes et al. 2017). Common signs include abdominal pain, nausea, vomiting, diarrhea and low-grade fever (Pellegrini et al. 2004; Ivanović et al. 2017).

In general, 1–2 weeks after the ingestion the inflammation becomes chronic (Audicana and Kennedy 2008; Baptista-Fernandes et al. 2017). At this stage, granulomatous lesions occur at the site of adhesion and hypersensitivity response is induced, producing immunoglobulin E (IgE) (Audicana and Kennedy 2008). Acute allergic reactions mediated by IgE may occur in previously sensitized patients (Pravettoni et al. 2012; Ivanović et al. 2017). Allergic symptoms, such as urticaria, angioedema and anaphylaxis, develop within 24 hours after the ingestion of contaminated food (López-Serrano et al. 2000; Foti et al. 2002; Bucci et al. 2013). Tissue eosinophilia is recognized in both acute and chronic inflammation stages of anisakiasis (Audicana and Kennedy 2008). An elevated eosinophil count in the damaged tissues is typical for an acute parasitic inflammation (Ivanović et al. 2017). Tissue eosinophilia is also associated with responses involving IgE production, which occurs both in acute allergic reaction and chronic inflammation stage of anisakiasis (Audicana and Kennedy 2008; Pravettoni et al. 2012; Ivanović et al. 2017).

In rare ectopic forms of anisakiasis, larva migrans can be found in the body cavity and surrounding organs, which causes chronic inflammation and symptoms related to the infected tissue (Hochberg and Hamer 2010; Pravettoni et al. 2012; Bucci et al. 2013). Severe cases of invasive anisakiasis may develop complications, such as peritonitis, intestinal obstruction and gastrointestinal perforation (Pellegrini et al. 2004; Hochberg and Hamer 2010; Takabayashi et al. 2014). In humans, larvae of *A. simplex* die and become spontaneously eliminated within three weeks of exposure (Audicana and Kennedy 2008; Pravettoni et al. 2012; Ivanović et al. 2017). However, inflamed lesions

may remain for weeks or months after the larval death, causing chronic symptoms (Mineta et al. 2006; Audicana and Kennedy 2008; Bucci et al. 2013).

2.1.3 Diagnosis, treatment and prevention

Diagnosis of anisakiasis is challenging, since the symptoms and diagnostic methods are nonspecific. If larvae are found in the sputum, vomit or stool of the patient, the disease may be diagnosed based on the morphological or molecular analysis of the nematode (Beaudry 2012). Endoscopic visualization and removal of the larvae is preferred method to diagnose and treat gastrointestinal anisakiasis (Mineta et al. 2006; Hochberg and Hamer 2010; Bucci et al. 2013). Removal of the larvae at the early stage of the infection usually relieves the symptoms and prevents the development of chronic manifestations. Diagnosis and treatment of severe invasive anisakiasis, especially in complicated cases, may require abdominal surgery (Pellegrini et al. 2004; Hochberg and Hamer 2010). Radiography and ultrasonography may be useful diagnostic aid in some cases. During laboratory examinations, leukocytosis may be present, especially when complications occur and peripheral eosinophilia may be present 8–15 days after the ingestion of the parasite (Pellegrini et al. 2004). Human infection with *A. simplex* leads to the production of specific anti-*A. simplex* antibodies (Hochberg and Hamer 2010; Ivanović et al. 2017). Development of immunoglobulin G (IgG), immunoglobulin A (IgA) and IgE occurs during the first month after initial infection (Hochberg and Hamer 2010). Elevated levels of total IgE are also typically observed with parasitic infections (Shimamura et al. 2016; Ivanović et al. 2017). Currently there are commercially available serological tests to measure anti-*Anisakis* IgG, anti-*Anisakis* IgA and *Anisakis* specific IgE titers (Shimamura et al. 2016). Unfortunately these tests lack high degree of sensitivity and specificity, and therefore cannot provide a definitive diagnosis alone. In uncomplicated cases, conservative therapy should be preferred instead of surgical intervention, since the larvae will eventually die in the human body (Pellegrini et al. 2004; Shimamura et al. 2016). Conservative treatment may include corticosteroids, antihistamines, fluid replacement therapy, pain control, antibiotics, proton pump

inhibitors or epinephrine (Foti et al. 2002; Bucci et al. 2013; Takabayashi et al. 2014). The larvae of *A. simplex* die within 10 minutes at the temperatures over 60°C and within 48 hours at the temperatures below −20°C (López-Serrano et al. 2000). However, recently cases of anisakiasis have been detected after the consumption of frozen fish in Spain (Gómez-Rincón C, personal notice 3rd October 2018). Therefore, the Spanish Agency for Consumer Affairs, Food Safety and Nutrition (AECOSAN) has extended the freezing time to five days at −20°C or below in domestic freezers. Comparably, the U.S. Food and Drug Administration (FDA) recommends the freezing time of seven days at the same temperatures (Centers for Disease Control and Prevention 2012a). Adequate processing of marine fish and squid is therefore the most efficient way to prevent anisakiasis infection.

Anisakiasis is likely an underdiagnosed disease since the symptoms are typical for many other gastrointestinal conditions. Patients with mild cases and spontaneous healing do not necessarily contact health care professionals. Current medical treatment occupies health care resources and causes discomfort for the patient. To eliminate the larvae, semi-invasive (endoscopy) or invasive (surgery) procedures must be performed. Manual removal of the larvae is time-consuming and demands special equipment and expertise. In addition, surgical treatment is always a risky and expensive operation. Even conservative treatment (as indicated in previous paragraph) should be performed under clinical observation, since it does not exclude the risk of complications (Shimamura et al. 2016). Currently, there is no proven pharmacological therapy for the treatment of anisakiasis. Pharmaceuticals provide only supportive therapy (e.g. by reducing pain, inflammation and allergic reaction), since there is no effective drug to kill the larvae once eaten (Bucci et al. 2013). In these circumstances, it would be beneficial to discover safe and effective compounds against *A. simplex* L3 larvae.

2.2 Nematodes

Life cycle of parasitic nematodes involves different hosts, which are essential for the development and maintenance of the parasites (Holden-Dye and Walker 2014; Partridge et al. 2018). Since the parasitic nematodes cannot maintain their life cycle under laboratory conditions, current research with *A. simplex* requires the isolation of the L3 larvae from fish. This procedure can be time-consuming, since the number of the larvae varies between each fish. In addition, this approach causes the waste of eatable fish and money. Clearly, other alternative models of *A. simplex* are needed.

C. elegans is a free-living nematode, which can be maintained in the laboratory (Blaxter 2011). The life cycle and the laboratory culture of *C. elegans* will be discussed in detail in Chapter 2.2.2. *C. elegans* has been widely utilized as a model organism in various research purposes (Holden-Dye and Walker 2014). This organism holds many advantages compared to the usage of *A. simplex* in the laboratory. *C. elegans* is inexpensive, easy to maintain in the laboratory and suitable for the study under a microscope (Donkin and Williams 1995; Blaxter 2011). It has a short life cycle and life span, which allows the fast production of large populations (Braeckman et al. 2000; Gershon and Gershon 2002; Blaxter 2011). Because of the small size of the nematode, these populations can be maintained in a small place (Blaxter 2011). In contrast to *A. simplex*, assays with *C. elegans* can be performed in a 96 or 384 well plate format, which allows the utilization of high throughput screening (hts) methods (Solis and Petrascheck 2011; Olmedo et al. 2015). These benefits evoke the interest in the possibility to use *C. elegans* as a model organism in the research of *A. simplex*.

2.2.1 Phylogeny

Nematodes (roundworms) constitute the phylum Nematoda, which is extremely large, diverse and widely distributed group of animals (Gilleard 2004; Holterman et al. 2006). The phylum contains both free-living worms (e.g. *C. elegans*) and parasites (e.g. *A. simplex*) which can be found in marine, freshwater and terrestrial environments. According to the latest molecular studies, there are 12 major clades in the phylum Nematoda (Holterman et al. 2006; Van Megen et al. 2009). Clades 1–2 (Enoplea) are considered as basal nematodes and Clades 3–12 (Chromadorea) as derived nematodes (Heger et al. 2009). These classes are further divided into three major branches (subclasses) of the phylogenetic tree, called Enoplia (Clade 1), Dorylaimia (Clade 2) and Chromadoria (Clades 3–12) (Holterman et al. 2006; Meldal et al. 2006; Van Megen et al. 2009). The evolutionary relationship of *A. simplex* and *C. elegans* is represented in Figure 1. *A. simplex* is placed in the Clade 8 and *C. elegans* in the Clade 9 of the phylogenetic tree (Heger et al. 2009; Park et al. 2011).

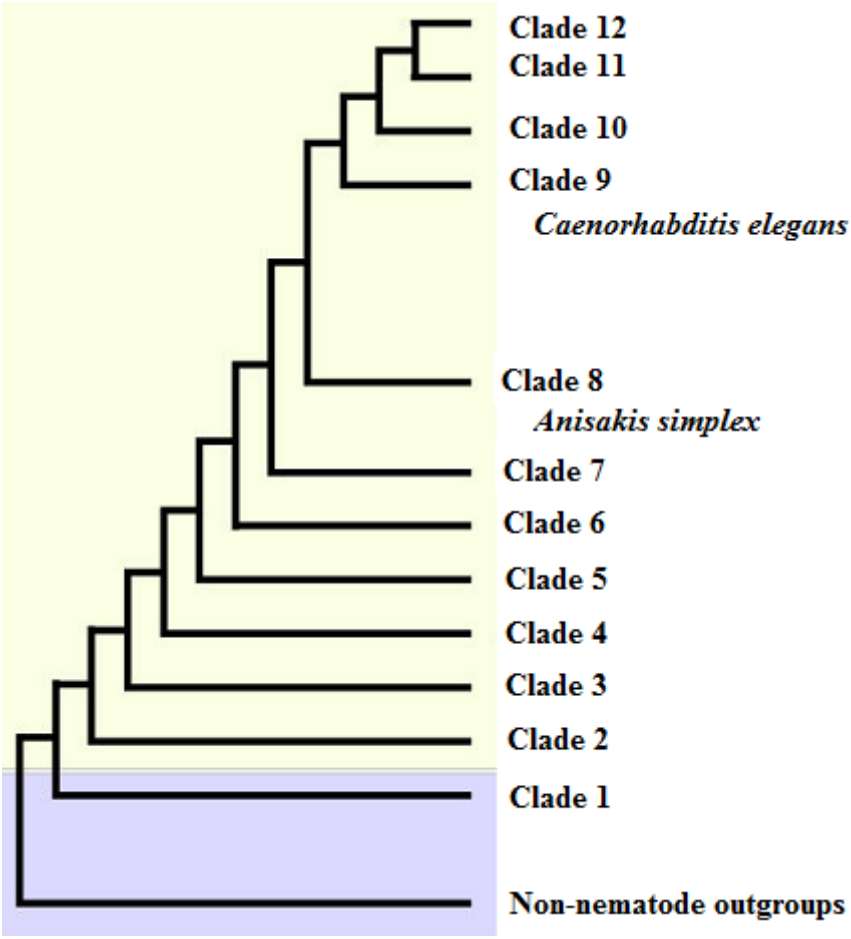


Figure 1. A simplified phylogenetic tree of the phylum Nematoda (modified from Heger et al. 2009). The phylum Nematoda contains 12 major clades: *Anisakis simplex* is placed in the Clade 8 and *Caenorhabditis elegans* in the Clade 9 (Holterman et al. 2006; Heger et al. 2009; Van Megen et al. 2009; Park et al. 2011).

2.2.2 Life cycle of *Anisakis simplex* and *Caenorhabditis elegans*

Development of *A. simplex* and *C. elegans* follows the basic nematode life cycle pattern. The life cycle stages include egg production, four larval stages (L1–L4) and adulthood (Klimpel and Palm 2011; Baptista-Fernandes et al. 2017). All nematodes molt at the end of each larval stage (Lee 1970). To increase size, larvae have to synthesize a new larger cuticle by the hypodermis. The old external cuticle is shed in the end of the molting process.

The life cycle of *A. simplex* is represented in Figure 2. Adult worms reside embedded in the gastric mucosa of marine mammals, where copulation and egg laying takes place (Pravettoni et al. 2012; Buchmann and Mehrdana 2016). Fertilized eggs are passed with host's feces to the sea and become embryonated in water (Pravettoni et al. 2012; Ivanović et al. 2017). First stage (L1) larvae are formed within the eggs and through molting they develop into free-living second stage (L2) larvae, which are released by hatching. Crustaceans act as intermediate hosts, in which maturation into L3 larvae occurs. Through predation the larvae are transferred to fish and squid, which act as paratenic hosts maintaining the infectious L3 population (Pravettoni et al. 2012; Baptista-Fernandes et al. 2017). The larvae migrate through the tissues of the paratenic hosts, found in the body cavity, viscera and muscle of fish and squid. L3 larvae of *A. simplex* are whitish to transparent with total length of 1–3 centimeters and width less than 1 millimeter (Figure 3) (Buchmann and Mehrdana 2016). By consuming contaminated, improperly processed seafood, humans become accidental hosts, in which the larvae cannot survive or progress to the adult stage (Shimamura et al. 2016; Baptista-Fernandes et al. 2017). Cetaceans act as natural final hosts, in which the larvae molt twice, developing into sexually mature males and females (Buchmann and Mehrdana 2016; Baptista-Fernandes et al. 2017).

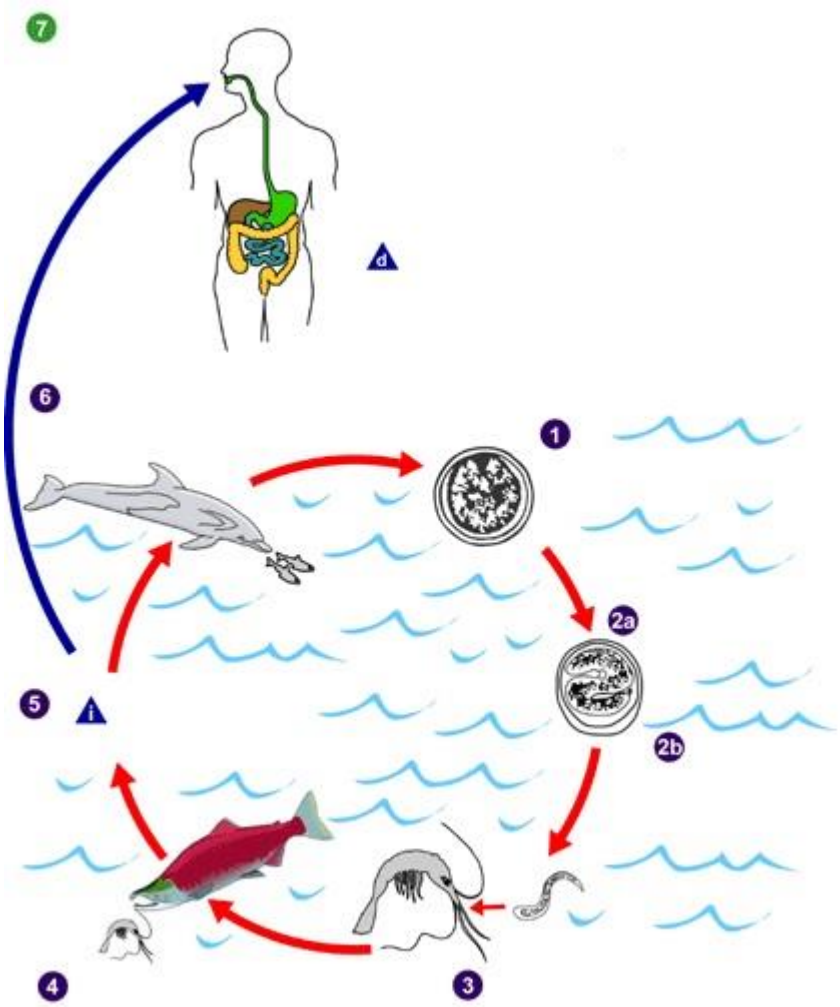


Figure 2. The life cycle of *Anisakis simplex* (modified from Centers for Disease Control and Prevention 2015). 1 = embryonation, 2a = formation of second stage larvae, 2b = hatching of free-living larvae, 3 = maturation into third stage larvae in crustaceans, 4 = larvae are transferred to fish and squid, 5 = maintaining of population through predation, i = infective stage, 6 = development of adult worms in marine mammals, 7 = humans become accidental hosts, d = diagnostic stage.

Figure 3. *Anisakis simplex* third stage larvae collected from blue whiting (*Micromesistius poutassou*).

C. elegans is a transparent, small (1–1.5 mm in length), free-living nematode found widespread in the soil (Gershon and Gershon 2002; Blaxter 2011). The natural food sources of the nematode include microorganisms, micronutrients and decaying organic matter, which is a source of plant sterols (Gershon and Gershon 2002; Fielenbach and Antebi 2008). The life cycle of *C. elegans* is represented in Figure 4. *C. elegans* occurs mostly as self-fertilizing hermaphrodites (Braeckman et al. 2000; Gershon and Gershon 2002; Blaxter 2011). In the fourth stage (L4) larvae, the germ cells develop as sperm and in the adult nematodes the germ cells develop as oocytes (Braeckman et al. 2000; Blaxter 2011). Egg-laying occurs typically before embryogenesis is completed. At room temperature L1 larvae hatch within 24 hours after the fertilization. Males are produced spontaneously at a low rate of approximately 0.1–0.2% (Braeckman et al. 2000; Gershon and Gershon 2002). However, males are able to mate with hermaphrodites and the resultant progeny consists of males at a frequency of 50%. Under unfavorable conditions of limited food, overcrowding or high temperature, the L1 larvae enter an alternate developmental pathway (Blaxter 2011). This will lead to the formation of lipid-storing alternate L2 larvae, which will further develop into diapausal L3 larvae, called dauer. Development of the dauer diapause is neurally controlled event induced by dauer pheromone (Braeckman et al. 2000; Gershon and Gershon 2002). Compared to the regular L3 larvae, the dauer larvae are much thinner and denser (Braeckman et al. 2000). They are non-feeding, non-aging and they have higher resistance against environmental stress, such as harsh chemical conditions (Braeckman et al. 2000; Blaxter

2011). The dauer larvae can survive for several months, having at least 8–10 times longer life span compared to the normal adult nematodes (Braeckman et al. 2000; Gershon and Gershon 2002). In favorable environment, the dauer larvae recover to the normal life cycle and life span (Gershon and Gershon 2002). To survive without feeding, *C. elegans* has to use lipid energy reserve during the diapause stage (Braeckman et al. 2000; Gershon and Gershon 2002). Thus, the depletion of the fat stores may lead to the death of the dauer larvae (Gershon and Gershon 2002).

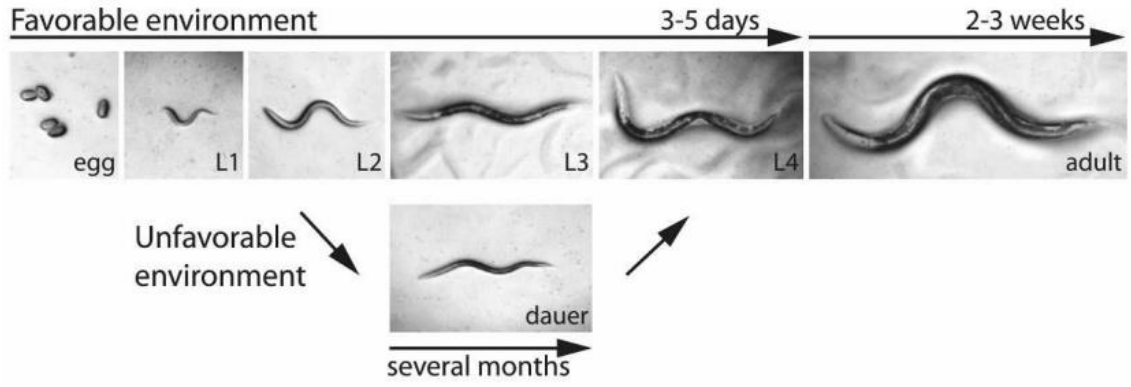


Figure 4. The life cycle stages of *Caenorhabditis elegans* (Fielenbach and Antebi 2008). In favorable environment, development from embryo through four larval stages (L1–L4) to adult nematode occurs within 3–5 days. Adult nematodes live 2–3 weeks. In unfavorable environment, a specialized third stage larva (dauer) is formed. Dauer larvae can survive several months and in favorable environment recover to the normal life cycle and life span.

In the laboratory, *C. elegans* is typically cultivated in monoxenic culture containing *Escherichia coli* (*E. coli*) cells, salts and cholesterol (Braeckman et al. 2000; Gershon and Gershon 2002; Fielenbach and Antebi 2008). Since nematodes are not able to biosynthesize sterols, 5–10 µg/mL of cholesterol is added to the nutrient medium (Braeckman et al. 2000; Gershon and Gershon 2002). The life cycle of *C. elegans* in favorable laboratory conditions is called reproductive development (Fielenbach and Antebi 2008). In monoxenic culture and at room temperature (20–22°C), the length of the life cycle is about 3–3.5 days and the mean life span of the adult nematodes is 2–3 weeks (Braeckman et al. 2000; Fielenbach and Antebi 2008). The life cycle of an adult hermaphrodite involves a 3–5 days long reproductive phase, during which they produce

approximately 300 eggs. For research purposes (e.g. toxicity, life span and metabolic studies), age synchronous worms of *C. elegans* are often required (Donkin and Williams 1995; Braeckman et al. 2000; Solis and Petrascheck 2011).

A synchronous population of *C. elegans* can be obtained by using alkaline bleach to dissolve gravid adult nematodes (Stiernagle 2006). The bleach breaks the bodies of *C. elegans* but does not affect the egg shells and the embryos inside. The axenized eggs are incubated in aquatic medium without food. During the overnight incubation, L1 larvae hatch but further development is arrested because of starvation. In nature, environmental fluctuations such as availability of food or changes in temperature, humidity, light exposure and oxygen tension, affect the development of *C. elegans* (Gershon and Gershon 2002). The nematode is also attacked by fungi and other organisms. However, in the laboratory *C. elegans* is cultivated under constant artificial conditions (Gershon and Gershon 2002; Blaxter 2011). This has led to the development of genotypes, which are less adapted to the environmental stress. Thus, it is important to be aware that the wild-type strain N2 used in the laboratory may not truly mimic the wild *C. elegans*.

2.2.3 Anthelmintic drugs

Anthelmintics are a group of drugs used for the treatment of animal infections caused by parasitic worms, including both flatworms and roundworms (Holden-Dye and Walker 2014). The majority of anthelmintic drugs have no cross-phyla activity, that is, they are either effective against flatworms (Platyhelminthes) or roundworms. Only benzimidazoles (e.g. albendazole) have activity against species in both phyla, although with higher efficacy against nematodes. There is limited evidence about the activity of albendazole against *A. simplex* L3 larvae. According to Dziekońska-Rynko et al. (2002) and Arias-Diaz et al. (2005), albendazole impaired the survival of *A. simplex* L3 larvae both *in vitro* and *in vivo*. Hence, some authors (Dziekońska-Rynko et al. 2002; Moore et

al. 2002; Arias-Diaz et al. 2005; Pacios et al. 2005) suggest that albendazole could be an effective anthelmintic drug for the treatment of anisakiasis. Daily dosages of 400–800 mg for 5–21 days have been used in clinical practice (Moore et al. 2002; Pacios et al. 2005; Baptista-Fernandes et al. 2017; Zanelli et al. 2017). However, the Finnish Medicines Agency (Fimea) and the FDA have not approved albendazole for this indication and in general anthelmintic compounds are not considered as an applicable choice for the treatment of anisakiasis (Beaudry 2012; Centers for Disease Control and Prevention 2012b; Shimamura et al. 2016; Terveysportti 2018).

2.3 Essential oils

Essential oils are natural products produced by various aromatic plants, which mainly appear in temperate to warm areas, including Mediterranean and tropical countries (Bakkali et al. 2008). To date, more than 3,000 essential oils have been discovered, of which approximately 300 oils are in commercial use. Generally, essential oils are used in pharmaceutical, agronomic, food, sanitary, cosmetic and perfume industries. Depending on the type of EO, antiseptic (i.e. bactericidal, virucidal and fungicidal), insecticidal and nematicidal properties have been reported (Bakkali et al. 2008; Valero et al. 2015). All plant organs (e.g. flowers, leaves, seeds, fruits, roots and wood) are able to synthesize essential oils as secondary metabolites, which are stored in secretory cells, cavities, canals, epidermal cells or glandular trichomes of the plant (Bakkali et al. 2008). Essential oils are characterized as liquid, volatile, clear and rarely colored compounds with strong odor. They are lipophilic compounds, which can be dissolved in lipids and organic solvents. Most of the essential oils have a lower density than water (ca. 1 mg/µL). Essential oils can be manufactured by several methods. Typically they are isolated from plant material by distillation, using hot steam or boiling water. Steam or hydro distillation can be performed either in low or high pressure. Other techniques include extraction with lipophilic solvents or supercritical carbon dioxide (CO_2). In some cases cold press extraction (i.e. expression) or microwave technology may be utilized (Carson et al. 2006; Bakkali et al. 2008). Environmental conditions and

manufacturing process have an effect on the final composition of EO (Bakkali et al. 2008). Therefore analytical monographs and composition standards (e.g. European Pharmacopoeia (Ph. Eur.) and International Standard (ISO)) have been published (Bakkali et al. 2008; Scientific Committee on Consumer Products 2008).

2.3.1 Composition

Chemically essential oils are complex mixtures of various compounds (Bakkali et al. 2008; Miguel 2010). The compositions of essential oils are typically determined by gas chromatography and mass spectrometry analysis. Most of the essential oils consist of approximately 20–60 components with different concentrations (Bakkali et al. 2008). Typically two or three major components are present with higher concentrations (20–70%) and the rest of the components are present in trace amounts. The components of essential oils can be divided into two groups according to the biosynthetic origin. The first group includes terpenes, which appear as hydrocarbons and their oxygenated derivatives (Bakkali et al. 2008; Miguel 2010). Oxygenated terpenes are also called as terpenoids (Bakkali et al. 2008). The second group consists of aromatic compounds. These two groups of molecules are biosynthesized from different origin through distinct metabolic pathways (Bakkali et al. 2008; Miguel 2010).

All components of essential oils have a low molecular weight (MW) (Bakkali et al. 2008). Terpenes can be divided into different classes based on their structure and functional properties. Terpenes are biosynthesized by combining isoprene (C_5H_8) units, which are generated through the mevalonate pathway (Carson et al. 2006; Bakkali et al. 2008). Monoterpenes (C_{10}) and sesquiterpenes (C_{15}) are the most common classes (Bakkali et al. 2008; Miguel 2010). Indeed, the vast majority (90%) of the constituents of the essential oils are monoterpenes with high diversity in chemical structures (Bakkali et al. 2008). The monoterpenes include hydrocarbons, alcohols, aldehydes, ketones, esters, ethers, peroxides and phenols. The two enantiomers of optically active

monoterpenes are typically found in different plants. However, some compounds (e.g. citronellol) occur mostly as a mixture of two enantiomers in the plants. The sesquiterpenes contain more extended carbon chain, which increases the cyclization of the molecules, resulting in the higher diversity in the structures. The sesquiterpenes include hydrocarbons, alcohols, ketones and epoxides. Hemiterpenes (C_5), diterpenes (C_{20}), triterpenes (C_{30}) and tetraterpenes (C_{40}) also occur, but with lower amounts. The aromatic compounds of essential oils are derived from phenylpropane, which is biosynthesized through the shikimate pathway. These compounds include aldehydes, alcohols, phenols, methoxy derivatives and methylenedioxy compounds. In addition to terpenes and aromatic compounds, essential oils generally contain aliphatic components in trace amounts. The secondary metabolites of aromatic plants may also include compounds, which contain nitrogen and sulfur in their structure (e.g. glucosinolates and their isothiocyanate derivatives). In most cases, the major components of the essential oils are responsible for the biological effects. However, most of the essential oils contain numerous molecules and therefore the synergistic effects cannot be excluded.

2.3.2 Essential oils used in the toxicity studies

In the experimental part of this thesis, Tea tree, Java citronella and Ho wood essential oils were used in the toxicity studies. Tea tree EO (CAS 68647-73-4) is mainly obtained from *Melaleuca alternifolia*, which is an Australian native plant (Carson et al. 2006; SciFinder 2018). It is manufactured from the leaves and terminal branches of the plant using steam distillation (Carson et al. 2006). Typical yield is 1–2% of the weight of wet plant material. Tea tree EO is colorless to pale yellow liquid with a relative density of 0.885–0.906 (European Pharmacopoeia 2018). It is sparingly soluble in water but can be dissolved in nonpolar solvents (Carson et al. 2006). Tea tree EO is composed of aromatic compounds and terpenes, containing mainly monoterpenes (80–90%) and sesquiterpenes (Carson et al. 2006; Scientific Committee on Consumer Products 2008). The major components are the monoterpenes terpinen-4-ol (30–48%) and γ-terpinene (10–28%). The main components of Tea tree EO according to International Standard

(ISO 4730-2004) are represented in Table 1. *In vitro* studies have revealed that Tea tree EO has antimicrobial (antibacterial, antifungal, antiviral and antiprotozoal) and antinematodal activity (Carson et al. 2006; Sfeir et al. 2013; Gómez-Rincón et al. 2014).

Table 1. Composition of Tea tree essential oil according to ISO 4730-2004 (Tighe et al. 2013). The percentage ranges of 15 main constituents are listed according to their relative abundance.

Constituent	**Percentage (%)**
Terpinen-4-ol	30–48
γ-Terpinene	10–28
1,8-Cineole	Trace–15
α-Terpinene	5–13
α-Terpineol	1.5–8
ρ-Cymene	0.5–8
α-Pinene	1–6
Terpinolene	1.5–5
Sabinene	Trace–3.5
δ-Cadinene	Trace–3
Ledene	Trace–3
Aromadendrene	Trace–3
Limonene	0.5–1.5
Globulol	Trace–1
Viridiflorol	Trace–1

Java citronella EO (CAS 8000-29-1) is obtained by steam distillation from the aerial parts of an aromatic citronella grass *Cymbopogon winterianus*, which originates from Sri Lanka (Wany et al. 2013; Dutta et al. 2016; SciFinder 2018). Java citronella EO is pale yellow to brown-yellow liquid with a relative density of 0.881–0.895 (European Pharmacopoeia 2018). The major components of Java citronella EO are the monoterpenes citronellal (30–45%), geraniol (20–25%) and citronellol (9–15%) (Bakkali et al. 2008; European Pharmacopoeia 2018). Other constituents include the monoterpenes geranyl acetate (3–8%), limonene (1–5%), citronellyl acetate (2–4%), geranial (max 2%) and neral (max 2%). Java citronella EO has shown *in vitro* antifungal, antibacterial, antinematodal and repellent activity (Pattnaik et al. 1996; Simic et al. 2008; Rani et al. 2013; Valero et al. 2015). Ho wood EO is obtained by steam distillation from the wood of *Cinnamomum camphora* (linalool chemotype), which is a native tree of Japan and Taiwan (Rabiul et al. 2011). The major component

of Ho wood EO is the monoterpene linalool (98.5%) (Sfeir et al. 2013). Ho wood EO has shown antibacterial activity *in vitro*.

2.3.3 Activity of essential oils against *Anisakis simplex*

Several essential oils have shown *in vitro* and *in vivo* activity against *A. simplex* L3 larvae. Essential oils caused morphological changes in the digestive tract, cuticle and muscular cells of the larvae (Romero et al. 2012; Giarratana et al. 2014; Romero et al. 2014; Gómez-Mateos Pérez et al. 2017). In the rat digestive tract wall, adherence and penetration of the larvae and development of the lesions were reduced (Romero et al. 2012; Romero et al. 2014; Gómez-Mateos Pérez et al. 2017). Giarratana et al. (2014) studied the activity of Thymol thyme (*Thymus vulgaris*) EO against L3 larvae of *A. simplex in vitro*. Concentrations of 0.1, 0.5, 1, 5 and 10% in sunflower oil caused complete inactivation (i.e. 100% mortality) after 120, 120, 96, 14 and 7 hours exposure, respectively. The treatment caused structural damages to the cuticle and digestive tract of the larvae. According to Valero et al. (2015), 125 µg/mL of Java citronella (*Cymbopogon winterianus*) EO produced 100% mortality in *A. simplex* L3 larvae after 48 hours exposure *in vitro*.

Romero et al. (2012) studied the activity of German chamomile (*Matricaria chamomilla*) EO against *A. simplex* L3 larvae *in vitro* and *in vivo*. A concentration of 125 µg/mL caused 100% mortality after 4 hours exposure *in vitro*. The treatment caused damage to the cuticle, digestive tract and muscular cells of the larvae. The EO was also effective *in vivo*, reducing the appearance of gastric wall lesions in infected rats. According to Romero et al. (2014), Peppermint (*Mentha* × *piperita*) EO was active against *A. simplex* L3 larvae both *in vitro* and *in vivo*. *In vitro* 250 µg/mL of the EO produced 100% mortality after 4 hours exposure and 187.5 µg/mL produced at least 83% mortality after 48 hours exposure. The treatment caused damage to the cuticle and digestive tract of the larvae. *In vivo*, the treatment with Peppermint EO prevented the

adherence of the larvae and development of the lesions into the gastrointestinal wall of infected rats. Gómez-Mateos Pérez et al. (2017) investigated the activity of different Mediterranean essential oils against L3 larvae of *A. simplex in vitro* and *in vivo*. According to their study, Oregano (*Origanum vulgare*) and Cumin (*Cuminum cyminum*) essential oils were active both *in vitro* and *in vivo*. The mortality rate of Oregano EO (5% volume/volume (v/v) in olive oil) after 24 hours exposure was 53.9% *in vitro*, causing severe oesophageal and intestinal damage in the larvae. The mortality rate of Cumin EO (5% v/v in olive oil) after 24 hours exposure was 20.3% *in vitro*. *In vivo*, treatment with Oregano, Cumin and Spanish lavender (*Lavandula stoechas*) essential oils prevented the penetration of the larvae into the digestive tract wall of infected rats.

Gómez-Rincón et al. (2014) examined the activity of Tea tree (*Melaleuca alternifolia*) EO against L3 larvae of *A. simplex in vitro*. A dose dependent activity was observed with the concentration range of 0.5–10 µL/mL. Concentrations greater than or equal to 5 and 4 µL/mL produced a significant ($p < 0.05$) lethal effect after 24 and 48 hours exposure, respectively. Of them, 10 and 7 µL/mL produced 100% mortality after 24 and 48 hours exposure, respectively. The median lethal dose (LD_{50}) value was 4.53 µL/mL at 24 hours and 4.27 µL/mL at 48 hours. López et al. (2018) studied the activity of Moroccan oregano (*Origanum compactum*) EO against *A. simplex* L3 larvae *in vitro*. A dose dependent activity was observed with the concentration range of 0–1 µL/mL after 24 and 48 hours exposure. A concentration of 1 µL/mL produced 100% mortality after 24 hours exposure. The LD_{50} values were 0.429 and 0.344 mg/mL at 24 and 48 hours, respectively. The EO also reduced the penetration ability of the larvae. According to Gómez-Rincón et al. (2014) and López et al. (2018), Tea tree EO and Moroccan oregano EO inhibited acetylcholinesterase, which is an important enzyme of the nematode neuromuscular function.

2.4 Coumarins

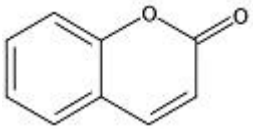
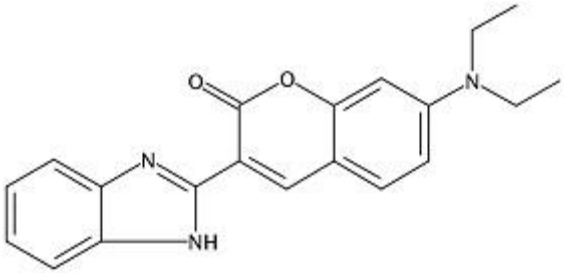
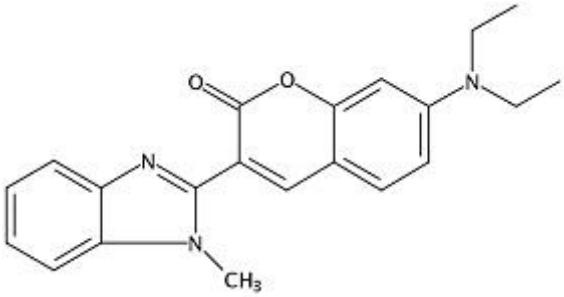
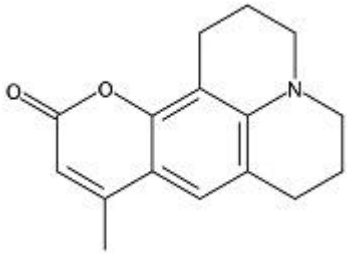
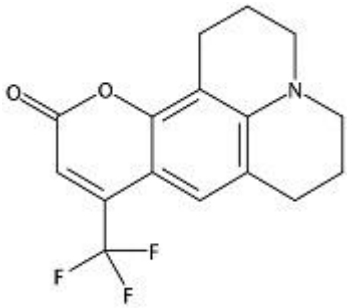
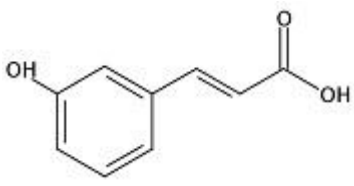
Coumarins are a large and widely distributed group of natural products, which are mainly produced by higher plants as secondary metabolites (Jain and Joshi 2012; Kumar et al. 2015). These aromatic compounds are derived from cinnamic acid, which is generated through the shikimate pathway. Coumarins are found in all parts of the plants, with highest amounts in fruits and seeds (Jain and Joshi 2012; Venugopala et al. 2013). Other coumarin rich parts include roots, stems and leaves. Some essential oils contain also high amounts of coumarins. Environmental conditions and seasonal changes may affect the amount of coumarins in different parts of the plant. Some coumarins are produced by microorganisms (bacteria and fungi) (Jain and Joshi 2012; Kumar et al. 2015). To date more than 1,800 natural coumarins have been identified (Kumar et al. 2015). Natural coumarins can be divided into different classes according to their chemical structure (Venugopala et al. 2013). Simple coumarins comprise of Coumarin (Table 2) and its derivatives with hydroxyl, alkoxyl or alkyl substituents in the benzene ring (Kumar et al. 2015). Furanocoumarins contain a furan or dihydrofuran ring and pyranocoumarins contain a pyran ring attached to the benzene ring of the parent compound (Venugopala et al. 2013; Kumar et al. 2015). Furano- and pyranocoumarins appear in linear or angular form. Phenylcoumarins belong to the class of coumarins with substituents in the pyrone ring and bicoumarins include coumarin dimers. Natural and synthetic coumarins have shown various biological activities, such as antibacterial, antifungal, antiviral and antinematodal activity (Venugopala et al. 2013; Pan et al. 2016). Coumarin and its derivatives are utilized in pharmaceutical, food, cosmetic, perfume, sanitary and laser industries among others (Kumar et al. 2015).

2.4.1 Coumarins used in the toxicity studies

In the experimental part of this thesis six coumarins were used in the toxicity studies (Table 2). Coumarin is the natural parent compound with the aromatic heterocyclic

structure of fused benzene and pyrone rings (Jain and Joshi 2012; Kumar et al. 2015). It is colorless crystalline powder characterized by a sweet odor. *m*-Coumaric acid is a natural product structurally classified as a hydroxyl derivative of cinnamic acid (Wiczkowski et al. 2016). C7, C30, C102 and C153 are coumarin derivatives with a dialkylamino substituent at carbon 7 (Table 2). This electron donating group together with an electron withdrawing lactone moiety brings intramolecular charge transfer (ICT) properties to the molecule (Sednev et al. 2015). These molecules are characterized by strong light absorption and subsequent emission. C7 and C30 contain a diethylamino substituent, whereas C102 and C153 contain a julolidyl ring in their structure (Table 2). In addition to the substituents at carbon 7, substituents at carbon 3 and 4 modify the properties of these compounds (Sednev et al. 2015). Depending on the electron withdrawing and donating character of the substituents at these positions, the wavelength and intensity of absorbed and emitted light may change. C7 and C30 contain a benzimidazole moiety at carbon 3 (Table 2). The hydrogen atom at nitrogen 1 position of benzimidazole is replaced by a methyl group in C30. C102 contains a methyl substituent at carbon 4, whereas C153 contains a trifluoromethyl substituent at the same position. The pattern of substitution of the parent coumarin structure has also an effect on the physicochemical properties, such as polarity, lipophilicity and solubility of the derivatives (Venugopala et al. 2013).

Table 2. Chemical structure of coumarins used in the toxicity studies (SciFinder 2018).

Compound	**CAS Number**	**Structural formula**
Coumarin	91-64-5	
Coumarin 7	27425-55-4	
Coumarin 30	41044-12-6	
Coumarin 102	41267-76-9	
Coumarin 153	53518-18-6	
m-Coumaric acid	588-30-7	

2.4.2 Activity of coumarins against nematodes

Several coumarins have shown activity against plant parasitic nematodes and free-living nematodes. Mahajan et al. (1992) investigated the activity of natural phenolic compounds with the concentration of 1100 ppm against *Meloidogyne incognita* (*M. incognita*) after 48 hours exposure. According to their study, compounds with coumarin moiety showed high activity against the nematode. Coumestrol and 7-hydroxycoumarin produced 100.0% and 90.3% mortality, respectively. Cinnamic acid derivatives showed also high activity. Among them, *m*-Coumaric acid produced 97.0% mortality. Wang et al. (2008) isolated three coumarins from the root extract of *Heracleum candicans* with activity against *Bursaphelenchus xylophilus* (*B. xylophilus*) and *Panagrellus redivivus* (*P. redivivus*). The median lethal concentration (LC_{50}) values of 8-geranyloxypsoralen, imperatorin and heraclenin at 72 hours against *B. xylophilus* were 188.3, 161.7 and 114.7 mg/L, respectively. The LC_{50} values of 8-geranyloxypsoralen, imperatorin and heraclenin at 72 hours against *P. redivivus* were 117.5, 179.0 and 148.7 mg/L, respectively. Liu et al. (2011) examined the activity of psoralen isolated from *Ficus carica* leaf extract against *B. xylophilus*, *P. redivivus* and *C. elegans*. The LC_{50} values at 72 hours were 258.8, 181.1 and 119.40 mg/L respectively.

Takaishi et al. (2008) and Pan et al. (2016) studied the activity of various synthetic coumarins against plant parasitic nematodes. Takaishi et al. (2008) synthesized alkoxycoumarins which showed activity against *B. xylophilus*. Among the tested compounds, 5-ethoxycoumarin showed the highest activity with the minimum effective dose (MED) of 10 µg/cotton ball (µg/bl.) after 96 hours exposure. Pan et al. (2016) synthesized coumarin analogs which showed activity against five plant parasitic nematodes. Among the tested compounds, 7-(4-bromobutoxy)-4-methylcoumarin showed the highest broad spectrum activity against all five nematodes. The LC_{50} value at 72 hours was 2.5 µM against *B. xylophilus*, 5.1 µM against *M. incognita*, 3.7 µM

against *Ditylenchus destructor* (*D. destructor*), 6.4 µM against *Bursaphelenchus mucronatus* (*B. mucronatus*) and 3.1 µM against *Aphelenchoides besseyi* (*A. besseyi*).

3 AIMS OF THE STUDY

The experimental part of the thesis was divided into two parts. In the first part, activity of tree essential oils (Tea tree, Java citronella and Ho wood) against *C. elegans* and *A. simplex* was examined. These essential oils have showed *in vitro* effectiveness against *A. simplex* L3 larvae (Gómez-Rincón et al. 2014; Valero et al. 2015; Gómez-Rincón C, personal notice 25th April 2016). The main purpose of the first part was to study whether the free-living nematode *C. elegans* could be used as a model organism for the parasite *A. simplex*. To our best knowledge, there is no previous research on this subject. The assays performed with *A. simplex* aimed at confirming the known activity of the essential oils. In the second part, activity of six coumarins (Coumarin, C7, C30, C102, C153 and *m*-Coumaric acid) against *C. elegans* and *A. simplex* was investigated. The main purpose of the second part was to discover novel active compounds against the pathogenic nematode *A. simplex*. The assays performed with *C. elegans* aimed at examining the possible comparable effects of active compounds.

4 MATERIALS AND METHODS

4.1 Chemicals

Tea tree (*Melaleuca alternifolia*) EO (Lot. 0F5075), Java citronella (*Cymbopogon winterianus*) EO (Lot. 0F12665) and Ho wood (*Cinnamomum camphora*) EO (Lot. 0F2381) were supplied by Pranarôm International. Nematode Growth Medium (NGM) with Cholesterol Supplement (Lot. L15020602DA) was purchased from bioWORLD. Luria Bertani Broth (LB Broth; Lot. BCBN9308V) was obtained from Sigma-Aldrich. Sodium Chloride (NaCl; Lot. 0000452435), Potassium Chloride (KCl; Lot. 0000337698), Potassium di-Hydrogen Phosphate (KH_2PO_4; Lot. 0000325514), di-Sodium Hydrogen Phosphate anhydrous (Na_2HPO_4; Lot. 0000321627), Cholesterol BioChemica (Lot. 4P017150), Magnesium Sulphate 7-hydrate ($MgSO_4 \cdot 7H_2O$; Lot. 0000437405), Ethanol absolute (99.5%; Lot. 0000538189) and Sodium Hydroxide pellets (NaOH; Lot. 0000391150) were acquired from Panreac. Sodium hypochlorite (14% Cl_2 in aqueous solution; Lot. 14I080503) was produced by VWR Chemicals. Coumarin, C7, C30, C102, C153 and *m*-Coumaric acid were acquired through Sigma-Aldrich.

4.2 Culture of *Caenorhabditis elegans*

C. elegans wild-type (N2) strain and *E. coli* OP50 strain were acquired from the *Caenorhabditis* Genetics Center (CGC), University of Minnesota, the United States. Maintenance and synchronization of *C. elegans* culture were performed in sterile conditions according to the standard protocols described by Stiernagle (2006), with some modifications. The worms were grown on NGM plates containing *E. coli* OP50 as a food source, at a temperature of 23°C.

4.2.1 Preparation of agar plates seeded with feeding bacteria

A colony of *E. coli* OP50 strain was inoculated into a test tube containing 5 mL of sterile LB Broth (Lennox formulation with Tryptone 10 g/L, Yeast Extract 5 g/L and Sodium Chloride 5 g/L). The bacterial suspension was incubated at 37°C for 24 hours and then stored in a fridge (2–8°C). Approximately 300–500 µL of *E. coli* suspension was pipetted at the center of a sterile NGM plate (60 mm diameter), which was then incubated at 37°C for 24 hours. NGM plates, with the final composition of Agar (17.5 g/L), Sodium Chloride (3 g/L), Peptone (2.5 g/L) and Cholesterol (5 mg/L), were prepared at least 24 hours before the seeding.

4.2.2 Preparation of a synchronous population

A chunk of agar (approximately 2 cm × 2 cm) was cut from an old NGM plate containing *C. elegans* wild-type (N2) strain and transferred to a clean NGM plate seeded with feeding bacteria. On the new plate the worms spread out onto the lawn of *E. coli* (Stiernagle 2006). Fresh plates with worms were incubated at 23°C for 48–72 hours. Plates containing many gravid adults were chosen for the synchronization. The plates were washed with sterile Millipore water (3.5 mL) and worms were collected into a 15 mL conical tube. A volume of 1.5 mL of freshly prepared mixture of 5 N NaOH and 5% NaClO (1:2) was added. To break the bodies of the worms and to release the eggs, the suspension was vortexed for 10–15 seconds every two minutes for a total of 10 minutes. The bleach also destroys the residues of *E. coli* and other possible bacterial or yeast contaminants (Stiernagle 2006). The suspension was centrifuged at 2620 rpm (revolutions per minute) for 2 minutes to form a pellet of eggs. Approximately 4.5 mL of supernatant was removed and 4.5 mL of sterile Millipore water was added. The eggs were washed by shaking and centrifuging at 2620 rpm for 2 minutes. Approximately 4.5 mL of supernatant was removed and 4.5 mL of sterile M9 buffer (1 mM $MgSO_4$, 22 mM KH_2PO_4, 42 mM Na_2HPO_4, 86 mM NaCl) was added. Possible residues of bleach

were neutralized by shaking and centrifuging at 2620 rpm for 2 minutes. Approximately 4.5 mL of supernatant was removed and the pellet was resuspended by pipetting.

Two methods were used for the seeding of the plates with worms. In the first method, the suspension was pipetted directly on an NGM plate seeded with feeding bacteria. The eggs were detected under a light microscope (Premiere®, Professional Microscope Binocular, MRP-5000) and the plate was incubated at 23°C for approximately 66 hours. After incubation, a synchronous population of adult individuals was detected under the light microscope. In the second method, the suspension was pipetted into a 50 mL conical tube containing 40–45 mL of sterile M9 buffer. The eggs were incubated horizontally with agitation (300 rpm) at 23°C. After 18 hours of incubation, the conical tube was held vertically in an ice bath for 15 minutes to allow the larvae to settle. Most of the supernatant was discarded, since it may contain dauer pheromone which has accumulated during starvation (Stiernagle 2006). Approximately 10 mL of M9 buffer was left on the bottom of the conical tube. The worms were resuspended by shaking and the suspension was poured into a 15 mL conical tube and centrifuged at 2480 rpm for 2 minutes. Approximately 9.5 mL of supernatant was removed, the pellet was resuspended by pipetting and the suspension was pipetted on an NGM plate seeded with feeding bacteria. The larvae were detected under the light microscope and incubated at 23°C for 48 hours. After incubation, a synchronous population of adult nematodes was detected under the light microscope.

4.3 Toxicity studies with *Caenorhabditis elegans*

An NGM plate containing a synchronous population of adult *C. elegans* was washed with 2 mL of sterile K-medium (32 mM KCl, 51 mM NaCl) and the suspension was pipetted into a 2 mL Eppendorf tube. The number of worms in 10 µL was determined under the light microscope (average of five volumes). Acute toxicity assays were

performed according to the method described by Donkin and Williams (1995), with some modifications.

4.3.1 Assays performed in 96 well plates

A stock solution (100 µL/mL) and test solutions (2.5, 5, 25, 50, 60 and 75 µL/mL) of Tea tree EO were prepared in sterile K-medium. The stock solution was directly used to prepare the final concentration 20 µL/mL. Approximately ten worms were introduced in a well of a sterile, flat-bottomed polystyrene 96 well plate containing K-medium with a final volume of 200 µL. Six wells of a column were used for each concentration and every second column was used to avoid mixing of different concentrations. A volume of 50 µL of Tea tree EO test solution was pipetted into the wells, acquiring the final volume of 250 µL in each test well. Six wells served as negative control, containing approximately ten worms in 250 µL of sterile K-medium. The control wells were prepared on the separate plate to avoid contamination with the volatile EO. The plate was sealed with a sticker and a lid and agitated at 300 rpm for 30 seconds. Test and control plates were incubated at 23°C for 24 hours. A percentage of dead worms per well was determined under the light microscope. Immobile worms were considered dead. The final concentrations of Tea tree EO were 0.5, 1, 5, 10, 12, 15 and 20 µL/mL. Total number of worms used to test every concentration was 185, 288, 308, 358, 61, 146 and 149, respectively.

4.3.2 Assays performed in Eppendorf tubes

Toxicity of Java citronella EO, Ho wood EO, Coumarin, C7, C102 and C153 against *C. elegans* was assayed using 1.5 mL Eppendorf tubes. Stock solutions and test solutions of essential oils were prepared in sterile K-medium. Three stock solutions of Java citronella EO were used to prepare the test solutions. A stock solution of 20 µL/mL was

used to prepare the test solutions of 0.5, 1, 4 and 8 µL/mL. The stock solution was directly used to receive the final concentration 5 µL/mL. A stock solution of 240 µL/mL was used for test solutions of 24, 36 and 48 µL/mL and a stock solution of 400 µL/mL was used for test solutions of 60, 72 and 80 µL/mL. The concentration of Ho wood EO stock solution was 20 µL/mL. Test solutions of 1, 2 and 4 µL/mL were used in the assay. Stock solutions of coumarins (20 mM) were prepared in DMSO (dimethyl sulfoxide) and test solutions were prepared in sterile K-medium. The concentrations of the test solutions were 80, 160 and 240 µM. Approximately 100 worms were introduced in an Eppendorf tube in K-medium with the final volume of 150 µL. A volume of 50 µL of test solution was pipetted into the worm suspension, acquiring the final volume of 200 µL. Two Eppendorf tubes were used for each test concentration. Two Eppendorf tubes served as negative control, each containing approximately 100 worms in 200 µL of sterile K-medium. Eppendorf tubes were incubated horizontally with agitation (200 rpm) at 23°C for 24 hours. A percentage of dead worms in a volume of 10 µL were determined under the light microscope. The mortality was determined in five drops of 10 µL from each Eppendorf tube (ten drops per concentration). Immobile worms were considered dead.

The final concentrations of Java citronella EO were 0.125, 0.25, 1, 2, 5, 6, 9, 12, 15, 18 and 20 µL/mL. The population size exposed for the concentrations 0.125, 0.25, 1, 2, 5, 6, 9 and 12 µL/mL was approximately 200 worms and for the concentrations 15, 18 and 20 µL/mL approximately 600 worms. For Ho wood EO, the final concentrations were 0.25, 0.5 and 1 µL/mL. The number of worms used to test every concentration was approximately 200. The final concentrations were 20, 40 and 60 µM for Coumarin and C153. For C7 and C102, the final concentration was 20 µM. Approximately 200 worms were exposed for each concentration of coumarins.

4.4 Toxicity studies with *Anisakis simplex*

Toxicity studies with *A. simplex* were performed according to the method described by Gómez-Rincón et al. (2014). Toxicity of Coumarin, C7, C30, C102, C153, and *m*-Coumaric acid against *A. simplex* L3 larvae was examined using flat-bottomed polystyrene 6 well plates. A 20 mM stock solution of each coumarin was prepared in DMSO and test solutions (40, 60, 80 and 120 µM) were prepared in sterile saline solution (0.9% NaCl). *A. simplex* L3 larvae were collected from blue whiting (*Micromesistius poutassou*) which were purchased from fish markets in Zaragoza, Spain. Larvae were washed with sterile saline solution and identified by macroscopic inspection. Ten larvae were introduced in each well of a 6 well plate containing 1 mL of sterile saline solution. A volume of 1 mL of test solution was added and three wells were used for each test concentration. Three wells served as negative control, each containing ten larvae in 2 mL of sterile saline solution. Test plates were covered with a lid and incubated at 37°C. An average mortality was determined after 24 and 48 hours. Immobile larvae were considered dead. The final concentration of Coumarin, C30 and *m*-Coumaric acid was 20 µM. The final concentrations were 20, 30 and 40 µM for C7 and C102. For C153, the final concentrations were 20 and 60 µM.

Toxicity of Tea tree EO (10 µL/mL) and Java citronella EO (5 µL/mL) was also examined using the same method. Test solutions (20 µL/mL of Tea tree EO and 10 µL/mL of Java citronella EO) were prepared in saline solution. In both assays, 20 larvae were exposed for test concentration and 20 larvae served as negative control. Controls were prepared on separate plates to avoid contamination with the volatile essential oils. An average mortality was determined after 24 hours incubation.

4.5 Data processing and analysis

Data processing and analysis was performed using Microsoft Excel software (version 2016).

5 RESULTS

5.1 Toxicity studies with *Caenorhabditis elegans*

Results of the toxicity studies with *C. elegans* are represented in Tables 3–6. Tea tree EO showed dose dependent lethal effect on *C. elegans* with the tested concentrations after 24 hours exposure (Table 3). The concentration 20 µL/mL produced 100% mortality after 24 hours exposure. However, a relatively high variation was observed in the mortality produced by the lower concentrations. The higher average mortality is associated with the higher standard deviation. The assays with Tea tree EO were performed in a 96 well plate format. Dead control worms were observed only in the assay in which the control wells were prepared on the same plate with the test wells. The mortality of the control worms was probably caused by the evaporation of the volatile EO. The concentration 12 µL/mL produced also 100% mortality after 24 hours exposure. This result was discarded due to the small population size (61 worms).

Table 3. Activity of Tea tree essential oil (EO) against *Caenorhabditis elegans*. Results are expressed as average mortality ± standard deviation after 24 hours exposure.

Compound	**Concentration (µL/mL)**	**Average mortality (%) 24 h**	**Population size (number of worms)**
Negative control		0.6 ± 2.0	344
Tea tree EO	0.5	1.2 ± 3.5	185
	1	4.0 ± 6.2	288
	5	9.0 ± 12.6	308
	10	16.1 ± 21.6	358
	15	53.1 ± 49.3	146
	20	100	149

Two methods were applied in the synchronization of the worm populations used in the toxicity studies with Tea tree EO (see 4.2.2). In the first method, the eggs were seeded directly on the NGM plate containing feeding bacteria. In this method the hatched L1 larvae continue their growth immediately in the presence of food. In the second method, the L1 larvae hatch in the aquatic medium without food, which arrests the development until the food source is provided. The usage of these two methods may have caused variation in the developmental stages of the worms used in the toxicity studies. Some worms may have been young adults and other worms further in their life cycle. These developmental stages may have different susceptibility to the EO. In general, it was also noted that the first method (seeding of the eggs on an NGM plate) produced less mobile worms. The populations used in the other toxicity studies were produced by the second method (overnight incubation of the eggs in aquatic medium). These assays were performed in 1.5 mL Eppendorf tubes and the results were extrapolated.

Java Citronella EO showed no significant lethal effect on *C. elegans* after 24 hours exposure (Table 4). The results indicate similar variation as noted with Tea tree EO, that is, higher standard deviation was observed with higher mortality. Dead control worms were detected only in the assay of three lowest concentrations. Concentrations 0.125 and 1 µL/mL produced higher mortality than much higher test concentrations. It is possible, that the populations used to test the three lowest concentrations contained abnormal individuals, which died regardless of the EO. Also, three times larger populations (i.e. 600 worms compared to 200 worms) were used to test the three highest concentrations. The higher population size increases the reliability of the results.

Table 4. Activity of Java citronella essential oil (EO) against *Caenorhabditis elegans*. Approximately 200 worms were exposed for concentrations 0.125, 0.25, 1, 2, 5, 6, 9 and 12 µL/mL. Approximately 600 worms were exposed for concentrations 15, 18 and 20 µL/mL. Results are expressed as average mortality ± standard deviation after 24 hours exposure.

Compound	**Concentration (µL/mL)**	**Average mortality (%)**
		24 h
Negative control		0.5 ± 2.8
Java citronella EO	0.125	6.1 ± 12.7
	0.25	0
	1	16.1 ± 20.5
	2	0
	5	0
	6	1.1 ± 3.5
	9	0.9 ± 2.9
	12	0
	15	4.3 ± 9.3
	18	2.1 ± 6.4
	20	2.1 ± 7.8

Ho wood EO showed no lethal effect on *C. elegans* after 24 hours exposure with tested concentrations (Table 5).

Table 5. Activity of Ho wood essential oil (EO) against *Caenorhabditis elegans*. Approximately 200 worms were exposed for each concentration. No mortality was detected after 24 hours exposure.

Compound	**Concentration (µL/mL)**	**Mortality (%)**
		24 h
Negative control		0
Ho wood EO	0.25	0
	0.5	0
	1	0

The tested coumarins (Coumarin, C7, C102 and C153) showed no lethal effect on *C. elegans* after 24 hours exposure with tested concentrations (Table 6).

Table 6. Activity of four coumarins against *Caenorhabditis elegans*. Approximately 200 worms were exposed for each concentration. No mortality was detected after 24 hours exposure.

Compound	**Concentration (µM)**	**Mortality (%)** **24 h**
Negative control		0
Coumarin	40	0
	60	0
Coumarin 7	20	0
Coumarin 102	20	0
Coumarin 153	40	0
	60	0

5.2 Toxicity studies with *Anisakis simplex*

Results of the toxicity studies with *A. simplex* L3 larvae are represented in Tables 7–8. Tea tree and Java citronella essential oils produced 100% mortality after 24 hours exposure with the concentrations 10 and 5 µL/mL, respectively (Table 7).

Table 7. Activity of Tea tree and Java citronella essential oils against *Anisakis simplex* third stage larvae. Twenty larvae were exposed for each concentration. After 24 hours exposure 100% mortality was detected. EO = essential oil.

Compound	**Concentration (µL/mL)**	**Mortality (%)**
		24 h
Negative control		0
Tea tree EO	10	100
Java citronella EO	5	100

The tested coumarins (Coumarin, C7, C30, C102, C153 and *m*-Coumaric acid) showed no significant lethal effect on *A. simplex* after 24 and 48 hours exposure (Table 8). The concentration 20 µM of C7 produced 30% mortality after 24 hours exposure. After 48 hours exposure, the higher mortality (46.7%) was detected. However, this result was discarded, since high mortality (> 10%) of control larvae was observed after 48 hours exposure. It can be assumed that the larvae exposed for this concentration contained abnormal individuals, since the higher concentrations of C7 showed no activity. For the same reason (high mortality of control larvae after 48 hours exposure), the other values in parentheses were also discarded. Among the tested compounds, C102 showed the most promising antinematodal activity. C102 produced 13.3% mortality after 48 hours exposure with the concentrations 30 and 40 µM.

Table 8. Activity of six coumarins against *Anisakis simplex* third stage larvae. Thirty larvae were exposed for each concentration. Results are expressed as average mortality ± standard deviation after 24 and 48 hours exposure. Values in parentheses were discarded due to high mortality of control larvae (> 10%).

Compound	**Concentration (µM)**	**Average mortality (%) 24 h**	**Average mortality (%) 48 h**
Negative control		0	3.4 ± 9.0
Coumarin	20	0	6.7 ± 5.8
Coumarin 7	20	30 ± 0	(46.7 ± 15.3)
	30	0	3.3 ± 5.8
	40	0	0
Coumarin 30	20	0	0
Coumarin 102	20	3.3 ± 5.8	(26.7 ± 5.8)
	30	3.3 ± 5.8	13.3 ± 5.8
	40	0	13.3 ± 15.3
Coumarin 153	20	0	(20 ± 0)
	60	0	0
m-Coumaric acid	20	0	0

6 DISCUSSION

Anisakiasis is an emerging disease, partly due to the changes in eating habits, that is, increased consumption of raw and undercooked seafood products (Pravettoni et al. 2012). In addition, the improvement of the diagnostic methods and better awareness of the disease have had a positive impact on the number of reported cases (Ivanović et al. 2017). There are several factors that may contribute to the occurrence of the infections in future. Increasing consumption of seafood products is related to the population growth, travelling, diffusion of local food cultures, globalization of seafood industry and development of transport (Pravettoni et al. 2012; Baird et al. 2014; Ivanović et al. 2017). These global changes have an influence on the amount and type of consumed seafood products. The lack of effective drug for the anisakiasis and emerging number of diagnosed cases drive the interest in the discovery of active compounds against *A. simplex*. Effective methods are required to test various compounds for further drug development steps. In this line, the usage of *C. elegans* as a tool in the search of novel active compounds against *A. simplex* would enhance the screening process.

6.1. *Caenorhabditis elegans* as a model nematode

In this study, activity of Tea tree EO against *C. elegans* was assayed to compare the effect to the known activity against *A. simplex*. According to Gómez-Rincón et al. (2014), 10 µL/mL of Tea tree EO produced 100% mortality in *A. simplex* after 24 hours exposure. This result was confirmed in our study (Table 7). In addition, comparable activity was observed in our study in *C. elegans*. After 24 hours exposure 20 µL/mL of Tea tree EO produced 100% mortality in *C. elegans*. This result indicates that two times higher concentration is required to produce same lethal effect in *C. elegans* as observed in *A. simplex*. However, the correlation between lower concentrations did not follow similar relation. Approximately three times higher concentrations of Tea tree EO were required to produce same degree of mortality in *C. elegans* compared to *A. simplex*.

After 24 hours exposure, 2, 3 and 5 µL/mL of Tea tree EO caused 10, 18.3 and 52.3% mortality respectively in *A. simplex* (Gómez-Rincón et al. 2014). By comparison, concentrations of 5, 10 and 15 µL/mL of Tea tree EO produced 9.0, 16.1 and 53.1% mortality respectively in *C. elegans* (Table 3). Overall, these results show that significantly higher doses are required to produce similar effects in the mortality of *C. elegans* compared to *A. simplex*. Because of the inadequate concentration range, the LD_{50} value against *C. elegans* could not be determined based on our results.

Kearn et al. (2014) investigated the toxicity of fluensulfone against *C. elegans*. They discovered that higher doses were required to reduce the survival, motility and egg hatching of *C. elegans* compared to the doses that caused similar effects on *Meloidogyne* species. These results are consistent with our study, indicating that *C. elegans* is less vulnerable than the parasitic nematodes. Kearn et al. (2014) suggest that the inequality of effective concentrations could result from different pharmacokinetics (e.g. metabolism) of the nematodes. In addition, the molecular targets may have different sensitivities to the tested compound in different species. Differences in pharmacokinetics and molecular targets are also possible reasons for the inequality of effective concentrations between *C. elegans* and *A. simplex*. According to Gómez-Rincón et al. (2014), the possible mechanism of action of Tea tree EO against *A. simplex* is the inhibition of acetylcholinesterase. The enzyme inhibition leads to the spastic paralysis of nematodes, which is caused by the increased synaptic levels of the neurotransmitter acetylcholine at the neuromuscular junction (Holden-Dye and Walker 2014). Thus, it is possible that the acetylcholinesterases of *C. elegans* and *A. simplex* have distinct sensitivity to Tea tree EO. Pharmacokinetically there are two routes to the target tissues of the nematodes; drugs can be ingested or diffused across the cuticle. The main constituents of the nematode cuticle are proteins, of which more than 80% is collagen (Russel et al. 2011). The tough and flexible cuticle forms a significant permeability barrier for several drugs (Russel et al. 2011; Holden-Dye and Walker 2014). Hence, the lipophilicity of the drug affects the achieved concentrations in the target tissues (Holden-Dye and Walker 2014). In general, the test concentration of polar drug may be 1,000 times higher than the predicted affinity to the molecular target. Since

essential oils are lipophilic compounds, it can be assumed that they reach the target tissues with relatively low concentrations.

Similarities in the physiology and pharmacology of the nematodes are important in the model hopping approach of toxicity studies (Holden-Dye and Walker 2014). Therefore the evolutionary relationship of nematodes is a significant factor. It is assumed that *C. elegans* is more relevant model for the close relatives, especially for the nematodes of Clade 9 (Gilleard 2004). *A. simplex* is placed in the relatively close Clade 8 (see 2.2.1). Although *Meloidogyne* species are placed in further Clade 12, it is suggested that *C. elegans* could be a suitable model for these plant parasitic nematodes (Kearn et al. 2014; Noon and Baum 2016). As discussed previously, higher doses were required against *C. elegans* than against the parasites *A. simplex* and *Meloidogyne* species. The inequality of effective concentrations supports the hypothesis that comparable effects are related to the phylogenetic distance. It is also possible that the differences in developmental stages contribute to the lethal effect. According to Kearn et al. (2014), larval stages were more vulnerable than adult nematodes. In our study, the L3 larvae of *A. simplex* were more susceptible than the adult nematodes of *C. elegans* to Tea tree EO. In addition these two nematodes have very different life cycles. *C. elegans* is a free-living nematode which is able to survive in artificial conditions (see 2.2.2). In nature, L3 larvae of *A. simplex* parasitize fish and squid. Therefore, *A. simplex* may be more vulnerable outside the natural habitat than the highly adaptive *C. elegans*.

In this study, Java citronella EO showed no significant lethal effect on *C. elegans*. According to Valero et al. (2015), 125 µg/mL of Java citronella EO produced 100% mortality in *A. simplex* after 24 hours exposure. The concentration 125 µg/mL is equivalent to 0.125 µL/mL, since the density of the EO is approximately 1 mg/µL (see 2.3). This was the lowest concentration tested in our study against *C. elegans*. Based on our results with Tea tree EO, it was predicted that two to three times higher concentrations could produce lethal effects. Although a wide concentration range (i.e. from two-fold to 160-fold) of Java citronella EO was tested, no mortality was observed

(Table 4). In our study 5 µL/mL of Java citronella EO produced 100% mortality in *A. simplex* after 24 hours exposure (Table 7). Compared to this, the four times higher concentration 20 µL/mL was tested against *C. elegans* with no effect. These results indicate that Java citronella EO is nontoxic to *C. elegans*. It is possible that the pharmacokinetics or molecular targets of Java citronella EO are significantly different in *C. elegans* compared to *A. simplex*, resulting in decreased activity. Similarly, Ho wood EO produced no mortality in *C. elegans* with tested concentrations (Table 5). To our best knowledge, there is no published data about the toxicity of Ho wood EO against *A. simplex*, or access to the published data about the effect of its major component (linalool) against the parasite. However, only three low concentrations (range 0.25–1 µL/mL) were tested in our study and the possible lethal effects of higher concentrations cannot be excluded.

Three tested essential oils produced contradictory results about the suitability of *C. elegans* as a model for *A. simplex*. Results obtained with Tea tree EO indicate comparable effects. However, assays with Java citronella and Ho wood essential oils produced no comparable effects. Two methods were used in the toxicity studies with *C. elegans*. Assays with Tea tree EO were performed in the 96 well plate format and the results were determined by detecting the whole test population. In turn, assays with Java citronella and Ho wood essential oils were performed in 1.5 mL Eppendorf tubes and the results were extrapolated. Since no positive controls were used, it is difficult to compare whether these two methods are equally valid. In addition, there is only few previous data about the effects of Java citronella and Ho wood essential oils for comparison. As discussed previously, only one published effective concentration of Java citronella EO and no data of Ho wood EO against *A. simplex* were available. In contrast, the dose dependent activity with wide concentration range and LD_{50} value of Tea tree EO against *A. simplex* were reported (see 2.3.3). Because of the high variation in our results, it is recommended to repeat the assays with Tea tree EO. A more suitable concentration range should be tested to be able to determine the LD_{50} value for comparison. In addition, the usage of positive control is recommended in future studies. Instead of Java citronella and Ho wood essential oils, Moroccan oregano EO could be

tested. Comprehensive data about its dose dependent activity and LD_{50} value against *A. simplex* are available for comparison (see 2.3.3). In addition, Tea tree and Moroccan oregano essential oils have similar mechanism of action (inhibition of acetylcholinesterase). It is recommended to perform all assays with constant methods to avoid variation caused by different approaches.

Our study with Tea tree EO produced promising results about the possible use of *C. elegans* as a model nematode for *A. simplex*. These findings may encourage researches to perform new assays to obtain more valid data about the correlation between effective concentrations against the two nematodes. Positive results would offer a new tool (i.e. *C. elegans*) for the studies that focus on the search of novel active compounds against *A. simplex*. More useful, economic and ecological method could increase the interest of researches and pharmaceutical industry in the discovery of drugs active against *A. simplex*.

6.2 Antinematodal activity of coumarins

In this study, activity of six coumarins against *A. simplex* was examined. According to our results, Coumarin, C7, C30, C102, C153 and *m*-Coumaric acid have no significant lethal effect on the pathogenic nematode (Table 8). Four compounds were tested against *C. elegans* for comparison purposes. Coumarin, C7, C102 and C153 produced no mortality in the free-living nematode (Table 6). The results indicate that these coumarins have no significant antinematodal activity against the tested species with the tested concentrations after 24 and 48 hours exposure. Since no significant mortality was observed, comparable effects cannot be determined based on our results at 24 hours. It is worth noting, that only one to three concentrations (range 20–60 µM) of each compound were tested and no positive controls were used in the assays. In addition, the population size of *A. simplex* (30 larvae) was relatively small in each assay.

To our best knowledge, only *m*-Coumaric acid (MW = 164.16 g/mol) has shown previous antinematodal activity among the six tested compounds (see 2.4.2). However, in our study only one concentration (20 µM) against one nematode (*A. simplex*) was examined. Mahajan et al. (1992) tested a 335-fold concentration (1100 ppm is equivalent to 6.7 mM), which produced 100% mortality after 48 hours exposure against *M. incognita*. In our study, 20 µM produced no mortality during equal exposure time. Probably higher concentrations would have no lethal effect on *A. simplex* since the nematode is placed in Clade 8, which is relatively distant from the Clade 12 of *M. incognita* (see 6.1). On the other hand, higher concentrations may have activity against *C. elegans*, since comparable effects were observed between *C. elegans* and *Meloidogyne* species (Kearn et al. 2014). Other plant parasitic nematodes were also susceptible to various coumarins (see 2.4.2). Of them, *D. destructor* is placed in Clade 12 whereas *A. besseyi*, *B. xylophilus* and *B. mucronatus* are placed in Clade 10 (Noon and Baum 2016). Other susceptible species were *C. elegans* and *P. redivivus*, which is a free-living nematode from Clade 10 (Srinivasan et al. 2013). Due to the evolutionary distance, the nematodes of Clades 10 and 12 differ from *A. simplex* and *C. elegans* and therefore may be more susceptible to coumarins. Only one compound (psoralen) was tested against *C. elegans* in previous studies (see 2.4.2). The LC_{50} value was lower against *C. elegans* than against *B. xylophilus*, which suggests that *C. elegans* is more vulnerable than the plant parasitic nematode. This result is contrary to the findings of Kearn et al. (2014), which suggest that *C. elegans* is less susceptible than the plant parasitic nematodes. These comparable results obtained with different parasites (*B. xylophilus* and *Meloidogyne* species) of different clades (10 and 12) support the hypothesis that the impact of phylogenetic relationship should be studied case by case (Gilleard 2004).

The coumarin derivatives used in this study are structurally different from the known antinematodal compounds. Psoralen, 8-geranyloxypsoralen, imperatorin and heraclenin are linear furanocoumarins (Bourgaud et al. 2006; Wang et al. 2008). Antinematodal simple coumarins include 4-hydroxycoumarin and 7-hydroxycoumarin (Mahajan et al. 1992). Especially the phenolic hydroxyl group was important for the antinematodal

activity. In addition, carbon 4, 5 and 7 are important modification sites of coumarin skeleton (Takaishi et al. 2008; Pan et al. 2016). Alkoxy groups, quinolone moiety and bromine atoms were important structures of antinematodal coumarin derivatives. The coumarin derivatives used in our study contain substituents at important positions. All of them are substituted at carbon 7; C102 and C153 contain also substituents at carbon 4. However, this pattern of substitution (dialkylamino moiety and methyl or trifluoromethyl group) seems to have little impact on antinematodal activity. In our study, C102 showed the most promising although weak antinematodal activity. This may imply the positive impact of methyl group at carbon 4, since this structure is also found in some nematicidal coumarins (Pan et al. 2016). C7 and C30 are substituted at carbon 3, which seems to be less important modification site. However, the benzimidazole moiety is found in many anthelmintic drugs, including albendazole (Holden-Dye and Walker 2014). The parent compound Coumarin showed no significant antinematodal activity in our study. Apparently, the substituents have an important function, since the antinematodal coumarins contain simple to more complex moieties attached to the basic coumarin structure.

According to this study, coumarins have no significant activity against *A. simplex* L3 larvae. Previous studies have focused on the activity of coumarins against the plant parasitic nematodes (see 2.4.2). Our results imply that coumarins may not have activity against the human parasitic nematode. Considering these findings, coumarins may be more applicable as nematicides than as antinematodal drugs. The methyl group at carbon 4 may contribute to the antinematodal properties of coumarins based on the activity of C102 and some nematicides (Pan et al. 2016). According to our results coumarins have no lethal effect on *C. elegans*, although the activity of C30 and *m*-Coumaric acid was not determined. On the other hand, psoralen showed activity against the free-living nematode (see 2.4.2). Thus, structurally different coumarins may have very distinct effects on the same nematode. Coumarins constitute a large group of compounds with varied structures (see 2.4). Therefore the activity of coumarins, which structurally differ from the compounds tested in our study, against *A. simplex* and *C. elegans* cannot be excluded. In addition, the small population size of *A. simplex*, the

narrow concentration range and the lack of positive controls decrease the reliability of the results.

7 CONCLUSIONS

In this study, activities of Tea tree, Java citronella and Ho wood essential oils against *C. elegans* were investigated. These assays were performed to examine whether these compounds produce similar effects against *C. elegans* as previously observed against *A. simplex*. According to our study, Tea tree EO showed dose dependent activity against *C. elegans* producing 100% mortality after 24 hours exposure with the concentration 20 µL/mL. Significantly higher doses (two to three fold) were required to produce same degree of mortality in *C. elegans* compared to *A. simplex*. The differences of the effective concentrations between both models are probably caused by the differences in the biology of the nematodes, which result from the phylogenetic distance. Due to the variation in our results, new assays should be performed with Tea tree EO to confirm the comparable effects. In addition, the LD_{50} value should be determined for comparison. The results obtained with Tea tree EO indicate that *C. elegans* may be a useful model in the research of *A. simplex*. However, Java citronella and Ho wood essential oils showed no significant activity against *C. elegans*. Because of these contradictory results, further investigation about the suitability of *C. elegans* as a model for *A. simplex* is required. Since there is only limited data about the activity of Java citronella and Ho wood essential oils against *A. simplex*, these compounds could be replaced by the well examined Moroccan oregano EO in future studies.

Furthermore, activity of six coumarins against *A. simplex* was examined. The purpose of these assays was to discover novel active compounds against the pathogenic nematode for further drug development steps. Coumarin, C7, C30, C102, C153 and *m*-Coumaric acid produced no significant mortality in *A. simplex*. Although our study has some limitations, it is possible to conclude that these coumarins are not potential antinematodal compounds against the human parasite. Previously coumarins have shown activity against the plant parasitic nematodes (see 2.4.2). Thus, this class of compounds may be more applicable as nematicides than as antinematodal drugs. In future studies, the methyl substituent at carbon 4 should be considered as potential

antinematodal structure since this moiety is found in weakly active C102 and some nematicidal coumarins (see 6.2). Activity of four coumarins against *C. elegans* was also investigated to examine possible comparable effects. Coumarin, C7, C102 and C153 produced no mortality in *C. elegans* and correlation could not be determined based on these assays.

Currently there is no effective drug for anisakiasis, which can be classified as a global emerging disease (Baptista-Fernandes et al. 2017). Therefore, novel active compounds against the pathogen *A. simplex* are desperately needed for drug development purposes. The usage of *C. elegans* as a tool in the discovery of drugs active against *A. simplex* would enhance the screening process, which could increase the interest of researches and pharmaceutical industry in this subject. The results of this study and previous studies support to some extent this possibility but further research is still needed. We are aware that the phylogenetic relationship is an important factor in model hopping approach and each case should be evaluated individually (Gilleard 2004; Liu et al. 2011; Kearn et al. 2014).

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