Application of rosemary Rosmarinus officinalis extract as an anthelmintic agent against Monogenean parasite in common carp Cyprinus carpio

Doctoral Dissertation

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Dissertation submitted to Tokyo University of Marine Science and Technology in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Marine Sciences

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Declaration

I hereby declare that this thesis was composed by me as a result of my own investigation. This work it has not been submitted for any other degree in this or any other university.

Mehmet Arif Zoral

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More than three years have passed since I arrived in Japan. During these years, I sacrificed the most important things such as my family, love and happiness in my life for doing Ph.D. However, I learned many valuable experience and knowledge about life.

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"Science is the truest guide in life" (Hayatta en hakiki mürşit ilimdir) (Atatürk, 1924).

Mehmet Arif ZORAL

March 2018

Tokyo, JAPAN

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Summary

Summary

Cyprinids are one of the most farmed fish in fresh water ecosystems. At present, one of the biggest problems in intensive cyprinid species production is ectoparasitic diseases. They affect the health and productivity of cyprinids. Parasites can cause skin erosion, mechanical damage, immunosuppression, secondary infectionsa and chronic stress. In addition, they cause economic losses, which result from mortality in fish stocks and high treatment costs.

Natural products such as medicinal plants have alternative approaches for parasitic treatments. They have an antibacterial, antiparasitic, antipathogenic, antitumour, immunostimulant activites. In addition, they were reported to have low toxicity to fish and have fewer adverse environmental impacts. The aim of this study is to determined to find new effective candidate herb against fish parasites. In addition, effective doses are determined to treat infected fish by herb extract in bath and oral treatment methods. In the second part of this study, toxicology and pharmacokinetic studies were investigated during the herb extract treatment.

First part, I examined the ffects of some herbs, such as rosemary, clary sage and thyme leaf extracts against *Ichthyophthirius multifiliis* (protozoa), *Trichodina* spp. (protozoa), *Dactylogyrus minutus* (monogenea), *D. extensus* (monogenea) and *Argulus* spp. (arthropoda) *in vitro*. Rosemary extract showed strong antiparasitic activity against protozoa and monogenea parasites. Based on the result of screening, I focused on the effects of rosemary extract against monogenean parasites and conducted various experiments for applying herb to control parasitic diseases. In addition, I evaluated the efficacy of rosemary extract by immersion and oral method to prevent and control monogenean infestation. In last part, I examined the safety of rosemary extract against fish. Also, I investigated 1,8-Cineole component (active component of rosemary extract) in fish blood and skin mucus.

In vitro parasite survival experiments revealed that both of ethanol and aqueous rosemary extracts reduce parasite survival time in dose dependent manner and ethanol extracts of rosemary had higher anti-parasitic activity against the *Ichthyophthirius multifiliis*, *Trichodina* spp., *D. minutus* and *D. extrensus* than that of aqueous extracts. The pure component of rosemary extract obtained commercially used in *in vitro* experiments showed that 1,8-Cineole was the most toxic component against *D. minutus*. The parasite intensity and prevalence in fish exposed to 50 and 100 g aqueous rosemary solution/L water for 30 min were significantly lower than those in controls ($p < 0.05$). In oral treatment experiments, diets of *C. carpio* were supplemented with eight different concentrations of aqueous rosemary extract. The intensity of *D. minutus* was significantly less in fish fed for 30 days with feed containing 60, 80 and 100 ml aqueous extract/100 g feed than in control ($p < 0.05$). No abnormal behavior was observed in any experimental fish group in the 30 day feeding period. Together these results indicate that rosemary is a promising candidate for prevention and control of monogenean infection. Aqueous rosemary extract demonstrated lower toxicity against host, and although its anthelmintic activity was slightly inferior to that of the ethanolic extract, the aqueous extract is a relatively safe and beneficial method for practical control of monogenean parasites.

In order to examine the safety of rosemary aqueous extract, toxicological study were conducted in fish were fed experimental diets supplemented with 10, 20, 40, 80, 100 ml extract/100 g feed for 20 days. According to the histopathological examination, the notable histological changes were detected in liver and kidney. Atrophy and nuclear pyknosis of hepatocytes were caused in the groups of ≥ 20 ml aqueous extract/100 g feed administration. In kidney, granular degeneration lead to necrosis and vascular irregularity were observed in groups of ≥ 40 ml aqueous extract/100 g feed. It was estimated that the LOAEL of rosemary extract against fish was 20 ml aqueous extract/100 g feed by the hepatotoxic effect. These

findings indicate that high doses of rosemary extract in diet for 20 days can be damage to liver and kidney.

In plasma chemistry analysis, fish were fed three experimental diets: control (without extract), 10 ml and 80 ml rosemary extract/100 g of feed for 20 days. Serum samples were taken at the end of 10 and 20 days feeding. AST (aspartate aminotransferase) activity of rosemary diet groups was tended to higher than of control group although the difference was not significant. The number of specimens that showed higher AST activity was increased with dose dependent manner. The AST is indicator of hepatocytes damage in vertebrates. It is suggested that the results of AST activity reflected hepatotoxicity of rosemary extract obtained by histopathological examination. In other clinical biochemical parameters, there were no significant differences among the experimental groups. These results suggest that the liver function and kidney damage were not serious.

1,8-Cineole was detected in blood 80 ml aqueous extract/100 g feed for 1 day. Following oral administration, 1,8-Cineole level in blood was reached to peak (117.89 ± 3.47 ng/ml) at 60 min after administration. After that, the amount of 1,8-Cineole in blood decreased exponentially from 2 to 72 h. The elimination half-lives ($T_{1/2}$) of 1,8-Cineole was calculated as 248 min in blood. 1,8-Cineole were detected 15.08 ± 7.6 ng, 49.8 ± 41.1 ng and 60.9 ± 37.7 ng in 10 mg crude dried mucus 5, 10 and 20 days administration, respectively. These results indicate that components of rosemary extract, such as 1,8-Cineole, absorbed by oral administration and travel through the blood, and can be secreted to mucus.

In summary, rosemary extract and its active compound of 1,8-Cineole showed anthelmintic activity against monogenean parasite. Both of oral administration and immersion of rosemary extract were effective to cure monogenean parasitic infection. In addition, component of rosemary extract was absorbed by oral administration and travel through the blood and

secreted into mucus. Based on these results, I concluded that rosemary extract would be candidate anthelmintic agent that apply to control of parasitic infection of farmed fish. On the other hand, it was revealed that rosemary extract had hepatotoxity and nephrotoxity in histopathological study and its LOAEL might be lower than that of effective dose. Further studies are recommended to establish suitable treatment regime for practical use of rosemary extract to cure parasitic infection in farmed fish.

Chapter 1

General Introduction

Civilization began with the age of agriculture. The agriculture has been considerable for human life and it will continue to be important in the future. In early age of agriculture, human have started to tame some animal and plant species. Some of these domesticated animals are; goat, sheep, pig, chicken, horse and common carp. The common carp (*Cyprinus carpio*) is one of the most important fish species for history of world aquaculture. Asian societies have begun to culture the carp since approximately 3500 years ago in China. Until today, production of carp species has been regularly increased in aquaculture history (FAO, 2016).

Nowadays, cyprinid species production has become an important protein source and vital food for human. These are especially true in most developing countries. In addition, carp culture is more ecofriendly than other aquaculture species such as salmon and shrimp. Because, they are an omnivorous filter-feeders and they consume much less protein resources (meal or oil). For these reasons, the common carp is amongst the major economically important and preferred cultured fish species in teleost family. It is globally accounts for 10% of freshwater aquaculture production and cultured in approximately 100 countries. In 2013, total global common carp production was over 4 million tons. This production will be increased in the future. Because, the global population of human is going to reach 9 billion people by 2050 and aquaculture production will have to increase meet the demands of a booming population. The Food and Agriculture Organization (FAO) estimated that the aquaculture production will increase from 63.6 million tons in 2011 to approximately 150 million tons by 2030 (Bostock et al., 2010; FAO, 2016).

Day after day, the carp culture is becoming more popular. For this reason, carp culture has many economic advantages for aquaculturists. However, this development may also lead to

various problems in aquaculture industry. For example, aquaculturists encounter some problems such as water pollution, weather conditions, vandalism, escapes, expensive feed, wrong feeding strategy or formulation, residue risk and diseases in intensive, semi-intensive and extensive aquaculture (Woo, 2006; FAO, 2016).

Lack of optimal water conditions (temperature, pH, salinity, oxygen, ammonium, nitrite, etc.) or keeping high density of fish population can trigger stress factors that lead to the outbreak of various infectious diseases in intensive aquaculture. Infection diseases (bacterial, fungal, viral and parasitic) have become major problems in aquaculture industry. They have critical role in the success production of fish in intensive culture conditions. Diseases outbreaks can directly effect to huge economic losses in production. Hence, treatment, prevention and control of fish diseases therefore have been of critical concern in aquaculture (Woo, 2006; Bruno, 2014).

Recently, researchers have begun to research useful treatment or control methods and new therapeutic agents against various diseases for aquaculture industry.

Parasitic Diseases

The presence of parasitic infections in fish leads to serious problem in fish farms. Large number of protozoan and metazoan parasites species are distributed in worldwide. Various ectoparasite and endoparasite species such as *Ichthyophthirius multifiliis* (protozoa), *Trichodina* spp. (protozoa), *Ichthyobodo* spp. (protozoa), *Eimeria* spp. (protozoa), *Chilodonella* spp. (protozoa), *Henneguya* spp. (myxozoa), *Myxidium* spp. (myxozoa), *Sphaerospora* spp. (myxozoa), *Myxobolus* spp. (myxozoa), *Dactylogyrus* spp. (monogenea), *Gyrodactylus* spp. (monogenea), *Argulus* spp. (arthropoda), *Ergasilus* spp. (arthropoda), *Lernaea* spp. (arthropoda), *Tracheliastes* spp. (arthropoda), *Diplostomum spp.* (trematoda), *Clonorchis sinensis* (trematoda), *Opistochis felineus* (trematoda), *Sanguinicola* spp.

(trematoda), *Caryophillaeus* spp. (cestoda), *Ligula intestinalis* (cestoda), *Bothriocephalus acheilognathi* (cestoda), *Khawia sinensis* (cestoda), *Triaenophorus* spp. (cestoda), *Philometroides* spp. (cestoda), *Anisakis* spp. (nematoda), *Contracaecum* spp. (nematoda), *Camallanus* spp. (nematoda), *Philometra* spp. (nematoda), *Acuntocephala* spp. (nematoda) have been recorded from common carp (Woo, 2006; Bruno, 2014).

In this study, I examined and identified 5 ectoparasite species; *Ichthyophthirius multifiliis*, *Trichodina* spp., *Argulus* spp., *Dactylogyrus minutus* and *D. extensus*. As this reason, I would like to give detail information about these parasite species in introduction part.

Ectoparasite infection of common carp (Cyprinus carpio)

Ichthyophthirius multifiliis

Ichthyophthirius multifiliis is a ciliated protozoan parasites belonging to phylum of Ciliophora. Commonly, it has known as white spot or Ich. It causes significant economic losses to the ornamental (goldfish and channel catfish, etc.) and freshwater culture fish (rainbow trout and carp, etc.) (Woo, 2006; Bruno, 2014).

Parasites are localized on the skin, fins and gills of infected fish. They cause physiological effects and damage host's tissue. Ich causes lesions and ulcer. In additions, erosion in fins is observed in clinical examination. In histopathology examination of infected fish, increased mucus production was examined in gills and skin. They have a direct life cycle (Fig. 1.1). In throphont stage, parasites penetrate to host skin or gill and feed on the epithelia. When adult parasites (tomont) ready to reproduction, they leave from the host epithelia. Tomont secrete gelatinous cyst on the water floor. Into the cyst, tomont divide to form daughter tomites. After

the division is complete, theronts (free-swimming larva) bore through cyst wall and they can infect fish (Woo, 2006; Bruno, 2014).

Breaking the life cycle of parasites is an important factors for control the infections. The most vulnerable phase of the infection is the theront phase that floats freely in water. The simplest treatment is that daily transfer of fishes to new different aquarium and water system for 5-7 days. Thus, parasite life cycle is break and theronts cannot infect to fish. The other control methods are to use ultraviolet light, disinfection methods, increase the water temperature. Moreover, parasite is control by chemicals such as sodium chloride (7000-20000 ppm), formalin (160-250 ppm for 1 hour), potassium permanganate (2-5 ppm for 30 min) and malachite green (0.1 ppm for 3-4 days) (Woo, 2006; Bruno, 2014).

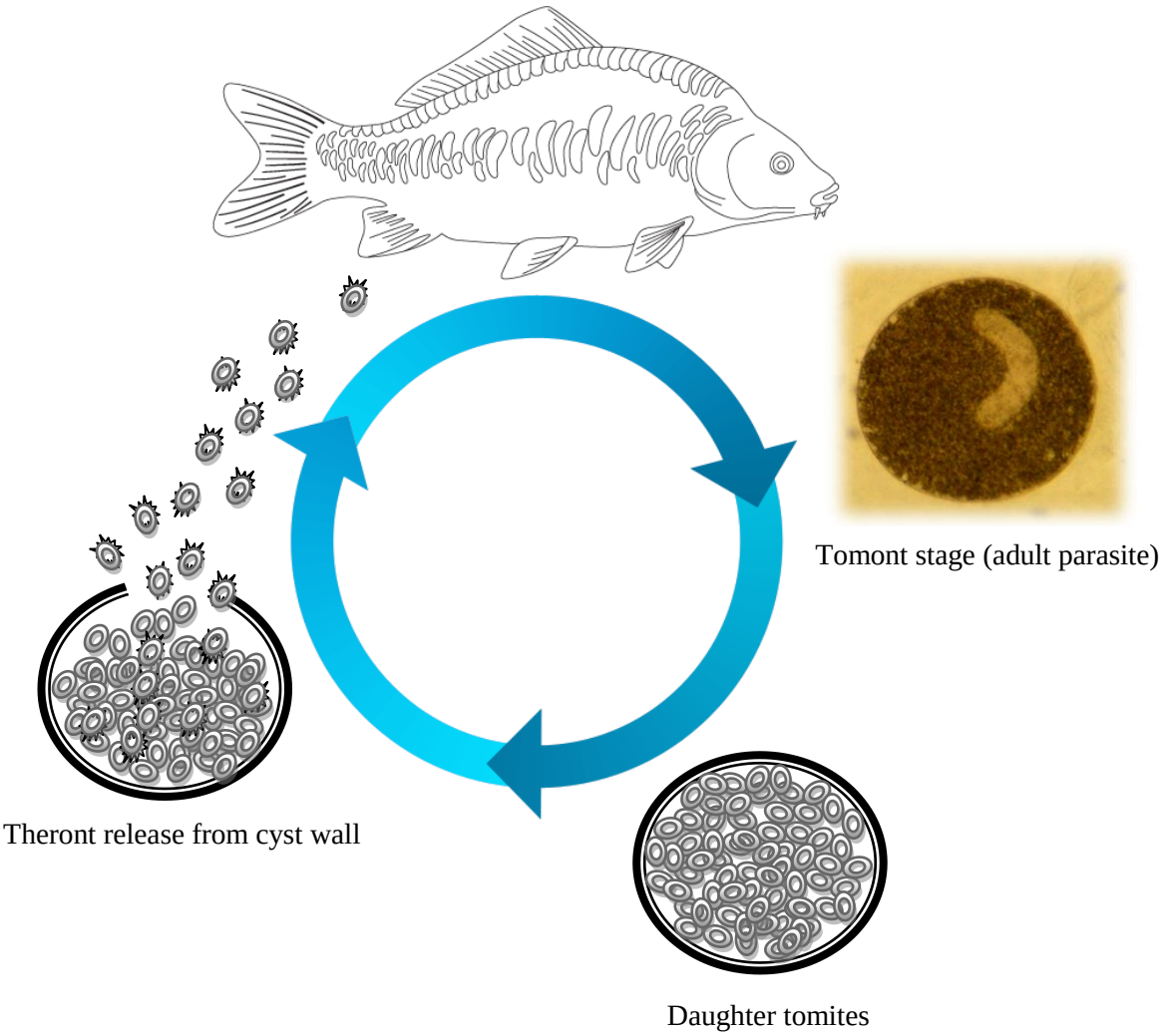

Fig. 1.1 Life cycle of *Ichthyophthirius multifiliis*.

Trichodina spp.

Trichodina spp. is ciliated protozoan parasites of marine and fresh water fish species (common carp, tilapia, rainbow trout, sea bream and sea bass etc.). They are one of the most common parasites in the worldwide (Woo, 2006; Bruno, 2014).

Parasite is located on the skin and gills. They cause serious pathological changes in the surface of the skin and gills. In heavy infections, fish are show lesions and ulcers in skin. In additions, they cause secondary infections (bacterial, fungal and viral diseases), stress, weight loss and mortalities. They have a direct life cycle and reproduce by binary fission (Woo, 2006; Bruno, 2014).

Keeping water parameters in optimum, compliance with cleaning protocols, improvement of feed quality and quarantine protocol for new fish stock have been the standard prevention strategies against parasite. Sodium chloride (in common carp 2% for 15 min at 30°C or 1% for 30 min at 20-25°C), formalin (150-250 ppm for 30-60 min), acriflavin (10-20 ppm), malachite green (2.5-5 ppm for 30 s), potassium permanganate (0.1% for 30-45 s) are used for treatment (Woo, 2006; Bruno, 2014).

Argulus spp.

Argulus spp. is metazoan ectoparasite in the phylum of arthropoda. It has known as fish lice. Most species can infect freshwater fish species such as carps, rainbow and brown trout. Some researchers reported that a few species were found in marine environments (Woo, 2006; Bruno, 2014).

They can cause significant morbidity and death in heavily infested fish. They feed on blood and epithelia. Infected fish are shown inflammation and corrosion in skin and fins. In addition, other clinical symptoms are stress, cease feeding activity and weight loss.

Researchers reported that parasite has been shown to serve as a vector for other diseases (bacterial and viral). They produce eggs for reproduction. Incubation time of egg depends on water parameters (temperature, salinity, pH) (Woo, 2006; Bruno, 2014).

They have a directly life cycle and specific for their host (Fig. 1.2) (Bostock et al., 2010).

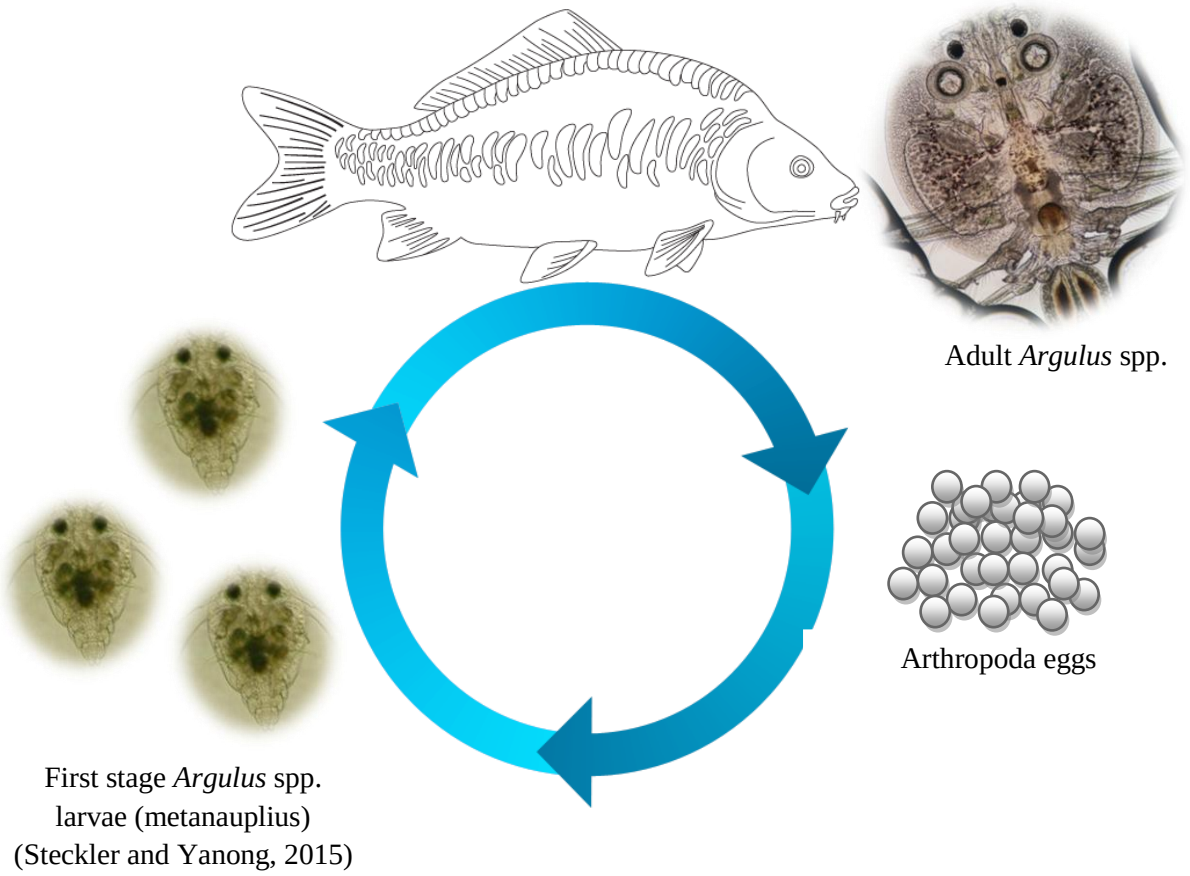

Fig. 1.2 Life cycle of *Argulus* spp. .

Previous studies reported that pesticide (gemmexane, etc.) and some commercial chemicals (azadirachtin, pyrethrum, dipterex, trichlorfon, neguvon, avermectins, doramectin, ivermectin and other antimalarial drugs) are used against the parasite in control and treatment methods. Piperine, a bioactive component of *Piper longum*, showed antiparasitic effects against

Argulus spp. (Kumar et al., 2012). In addition, biological and quarantine control methods are used for preventation (Woo, 2006; Hemaprasanth et al., 2012)

Monogenea

Monogenean parasite (Monopisthocotyleans and Polyopisthocotyleans) are Platyhelminthes (flatworms) of freshwater and marine farmed fish that cause significant economic losses. Monogeneans easily infect to fish and cause high mortality in poor water conditions. Most monogeneans are ectoparasites but a few species are endoparasites in bladder and urinary ducts organs. The majority of species are located in host's gills or skin. For example, species of *Dactylogyrus* spp. is only located in gills, while *Gyrodactylus* spp. is located in skin and gills (Woo, 2006; Bruno, 2014).

Monogenean feed on mucus and blood. They cause serious gill and body surface infestations results in problems such as tissue reactions, lethargy and cachexia. Moreover, monogenean may lead to secondary infections such as bacterial, fungal and viral diseases and besides some species such as *Pseudodactylogyrus bini* has been shown to serve as a vector for virus (Woo, 2006; Bruno, 2014).

They are a hermaphroditic organism. *Dactylogyrus* spp. is oviparous and free-swimming ciliated larvae (oncomiracidia) emerging from eggs can infect fish directly without an intermediate host (Fig. 1.3). Eggs become embryonated in marine or fresh water. Incubation time of eggs depends on water temperature, pH, salinity and other environment factors. Conversely, *Gyrodactylus* spp. is viviparous. They have a well-developed uterus and give birth to embryo. Temperature affect rate of reproduction (Woo, 2006; Bruno, 2014).

The standard treatment chemicals for monogonean are copper sulfate, potassium permanganate, hydrogen peroxide, trichlorfon and some commercial parasite drugs (praziquantel and mebendazole). Formalin applications (200-250 ppm for 30 min or 500 ppm

for 10 min) are effective for short-term or prolonged immersion treatment. In addition, several studies have been reported that aqueous and methanolic extracts of garlic (*Allium sativum*), aqueous and methanolic extracts of ginger (*Zingiber officinale*), methanolic extract of bupleurum root (*Radix bupleuri chinensis*), aqueous and methanolic extracts of cinnamon (*Cinnamomum cassia*), methanolic extract of Chinese spice bush (*Lindera agreggata*) and methanolic and ethyl acetate extracts of golden larch (*Pseudolarix kaempferi*) have shown to possess 100% in vivo efficacy against monogenean species (Militz et al., 2013; Reverter et al., 2014; Levy et al., 2015).

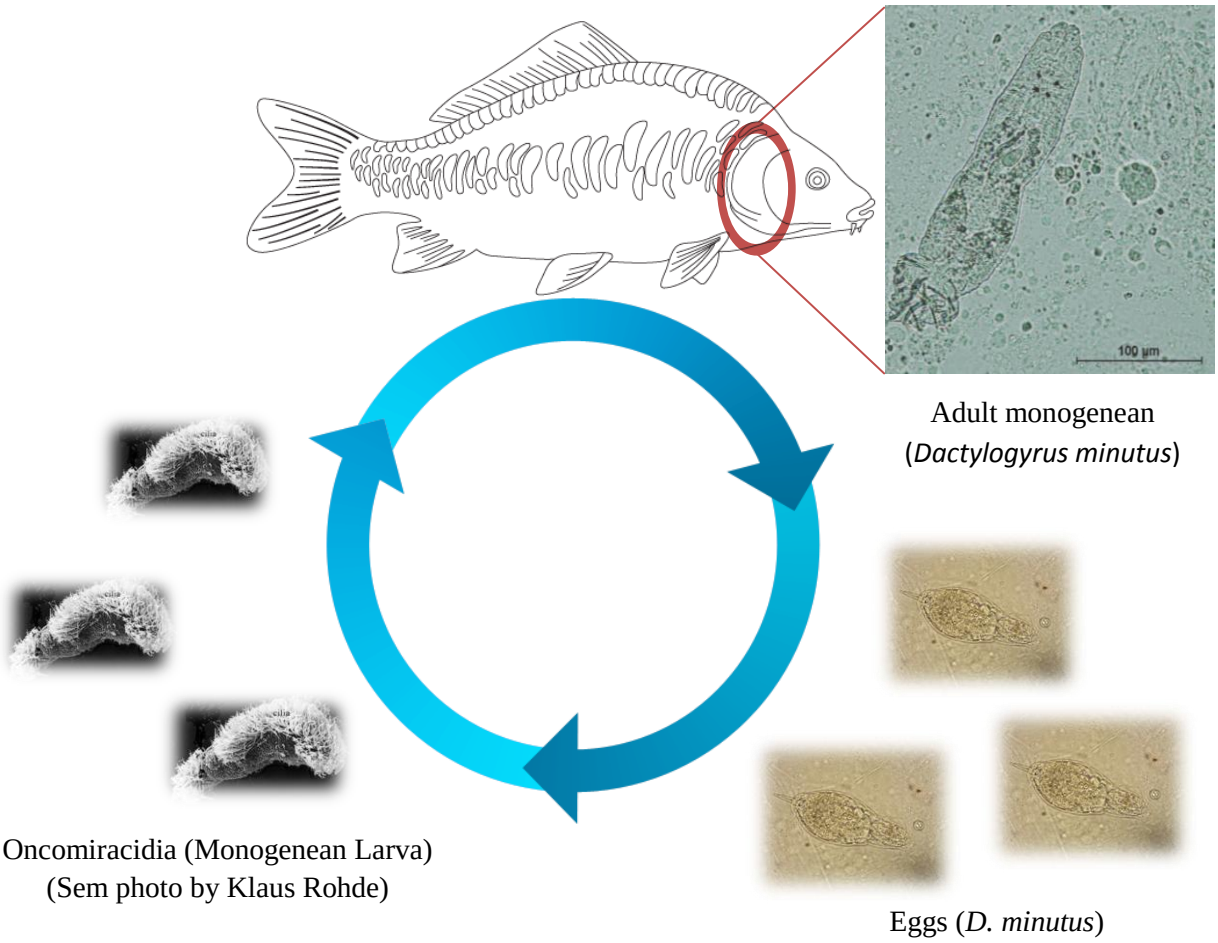

Fig. 1.3 Life cycle of *Dactylogyrus minutus*.

Herbal medicine in aquaculture

Traditional medicines include herbal medicines have been used to treat diseases for thousands of years. The herbal medicine has been relied upon to support, promote, prevention, control and regain human and animal health from past to present (Li et al, 2008; Shi et al., 2009).

Herbal remedies have become popular over the past decade in aquaculture. The herbs are an important source of bioactive compounds. Therefore, various herb extracts have been reported to have significant effects features such as antibacterial, antifungal, antiviral, antiparasitic, anti-stress, growth promotion, appetite stimulation, enhancement of tonicity, immunostimulation and maturation of culture species in fish. In recent years, usage of plant in aquaculture has been become effective and beneficial methods for prevent and control infections (bacterial, viral, fungal and parasitic). Moreover, use of chemicals such as formalin, copper sulfate, potassium permanganate, hydrogen peroxide, trichlorfon have multiple negative impacts. These negative impacts are usually causing toxic residues and stress in fish and pollute aquatic environment. For these reasons, recently, researchers have begun to find alternative new therapeutic agents against various diseases (Reverter, 2014).

Most plants are proposed as biological control agents in aquaculture belong to the garlic (*Allium sativum*), artemisia (*Artemisia capillaris*), black paper (*Piper guineense*), chinese cinnamon (*Cinnamomun cassia*), coriander (*Coriandrum sativum*), martius (*Cariniana legalis),* ginger (*Zingiber officinale*), rosemary (*Rosmarinus officinalis*), virola (*Virola sebifera*) and mistletoe (*Viscum album*) etc. (Table 1.1). In addition, previous a few studies reported that some herb and their extracts have been shown antiparasitic effects against parasite species in fish (Table 1.1). However, the most of these plants have not been adequately researched for their chemical composition and pharmacological properties in fish. It is therefore interesting to study these plants to identified active or effective components.

Furthermore, the detection of toxicity of these herbs must be confirmed in fish during the immersion or oral treatment. Every herb has the potential to cause harmful effects in living organism when given in the proper or effective doses for treatment (Citarasu, 2010; Reverter, 2014).

Table 1.1 *In vivo* antiparasitic studies of herbs extracts. (PE: petroleum ether, CHL: chloroform, E: ethanol EA: ethyl acetate, ME: methanol, B: Benzene).

Plant	Type of extract	Type of administration	Target Parasite Species	References
Allium sativum	Water (bulb) and powder (bulb)	Oral and Bath	*Neobenedenia* sp. *Dactylogyrus* spp.	Militz et al., 2013 Fridman et al., 2014
Artemisia annua	E	Bath	Monogenean	Ekanem and Brisibe 2010
Cimifuga foetida	ME, PE, CHL and EA	Bath	*Dactylogyrus intermedius*	Wu et al., 2011
Cinnamomum cassia	PE, ME, CHL, EA and water	Bath	*Dactylogyrus intermedius*	Ji et al., 2012
Dryopteris crassizhizoma	ME, PE, CHL, EA and water	Bath	*Dactylogyrus intermedius*	Lu et al., 2012
Hericium erinaceum	E	Oral	*Philasterides dicentrarchi*	Harikrishnan et al., 2011
Lippia sidoides	Essential oil	*in vitro*	Monogenean	Hashimoto et al., 2016
Macleaya cordata	ME	*in vitro*	*Gyrodactylus kobayashii*	Zhou et al., 2017
Mentha piperita	Essential oil	*in vitro*	Monogenean	Hashimoto et al., 2016
Santalum album	CHL, ME, EA and water	Bath	*Dactylogyrus intermedius* *Gyrodactylus elegans*	Tu et al., 2013
Semen aesculi	E	Bath	*Gyrodactylus turnbulli*	Liu et al., 2010
Sophora alopercuroides	ME	Bath	*Ichthyophthirius multifiliis*	Balasubramanian et al., 2007
Terminalia catappa	Water	Bath	*Trichodina* spp.	Chitmanat et al., 2005
Ulva sp.	Water	Bath	*Neobenedenia sp.*	Hutson et al. (012
Zingiber officinale	E	Bath	*Gyrodactylus turnbulli*	Levy et al., 2015

Herbs toxicity

Herb toxicity refers to the level of damage that active components of herb can cause to an organism. The toxic effects of herbs are dose-dependent and can affect an entire organs system as in a specific organ such as the liver and the kidney etc. Therefore, it's necessary to decide toxic and therapeutic doses of herbs for organs. Toxic and therapeutic effects may occur at the same doses in herb administration (Mohammed, 2012).

There are a limited number of scientific studies on the safety use of most herbs, and it is not enough well understood and searched in aquaculture. Many herbs contain constituents that could be allergic or toxic substance for internal organs. For example, *Aristolochia fangchi* diet was reported causing weight loss and nephropathy symptoms in human. Some patients developed end-stage renal failure with prohylactic kidney removal and urothelial carcinoma. In addition, diet of some herbs contains pyrrolizidine alkaloids, which cause veno-occlusisve diseases and then finally cause hepatotoxicity in liver (Bent, 2008). Other example, ephedra is widely used in traditional medicine for weight-loss and energy-enhancing products in China. The herb contains main active component of ephedrine. The clinical examination of ephedra was shown side effects such as risk of nausea, vomiting, psychiatric symptoms and palpitations compared with placebo in patients. As these reasons, using ephedra was banned by the Organization of Food and Drug Administration in United State of America (Bent, 2008).

Previous studies showed that aloe vera, bilberry garlic, ginger, ginseng, kelp storphanthus and yohimbine can cause some cardiovascular diseases such as increases bleeding risk with warfarin, increases heart rate, increases or decreases blood pressure, hypoglycemia and increases effects of cardiac glycosides (Bent, 2008).

The herb species are widely used for treatment of various diseases and healthy life. However, they can potentially be toxic. Because, they have an unknown organic chemicals, and their pharmacovigilance knowledge is not enough for using. Therefore, it is important to confirm their contained components, and safety doses for living organisms (Bent, 2008).

Aim of this research

Intensive aquaculture results in some serious parasitic diseases, affecting the health and productivity of the fish species. Ectoparasites of freshwater and marine farmed fish can cause significant economic losses. Chemical and synthetic drugs have been used to control parasitic infection in aquaculture. However, these drugs also leave harmful residues and adversely affect the environment and fish health. In recent years, parasitic disease management in aquaculture is concentrated on alternative approaches for effective and environmentally friendly treatments. Medicinal herbs can be potential treatment material against parasites in farm fish. They have been reported to possess some advantages for fish health. Therefore, the objective of this research is;

1. To search and determine new candidate herbs which have antiparasitic activity against the parasites (*Trichodina* spp., *Argulus foliaceus, Dactylogyrus minutus* and *Dactylogyrus extensus*) (Chapter 2);
2. To confirm antiparasitic activities of ethanolic and aqueous herbs extracts against *Dactylogyrus minutus* (monogenean) *in vitro* (Chapter 2);
3. To identify the antiparasitic active components in rosemary extracts *in vitro* (Chapter 2);
4. To reveal the safety levels of rosemary extracts in immersion for carp (Chapter 2);

5. To establish the practical procedures to treat infected fish with a monogenean parasite by immersion (Chapter 2);
6. To confirm the anthelmintic activity of aqueous rosemary extracts *in vivo* by oral administration (Chapter 2);
7. To examine the safety of rosemary aqueous extract *in vivo* against health fish by oral administration (Chapter 3);
8. To reveal the pharmacokinetics of 1,8-Cineole in rosemary extracts *in vivo* by oral administration (Chapter 3).

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Chapter 2

Section I

Antiparasitic activity of rosemary (*Rosmarinus officinalis*), clary sage (*Salvia sclarea*) and thyme (*Thymus capitatus*) against *Trichodina* spp., *Argulus foliaceus, Dactylogyrus minutus* and *Dactylogyrus extensus in vitro*

Abstract

The antiparasitic effect of rosemary, clary sage and thyme against *Trichodina* spp., *Argulus foliaceus, Dactylogyrus minutus* and *Dactylogyrus extensus* were examined by *in vitro* trials. The *in vitro* results showed that all herb extracts had antiparasitic effects against *Trichodina* spp. . In addition, it was observed that rosemary extracts had the strongest antiparasitic effect against *Dactylogyrus minutus* and *Dactylogyrus extensus*. However, antiparasitic effect was not observed against *Argulus foliaceus*, in all herb extracts. Together these results indicate that rosemary is a candidate herb for treatment of *Trichodina* spp. and *Dactylogyrus* spp. infections.

Introduction

The aquaculture sector has recently focused on alternative treatment methods and materials for parasitic diseases. Researchers have begun to investigate medicinal herbs, which are effective against parasitic diseases. In particular, this study has focused on the use of new candidate traditional medicinal herbs as an alternative control strategy against parasite *in vitro*.

Rosemary (*Rosmarinus officinalis*) is widely used to treatment in the Mediterranean area since ancient Greek times. It has a source among natural bioactive compounds due to its powerful antioxidant and anti-inflammatory activity, antibacterial, antimutagenic properties

and as a chemopreventive agent. In addition, rosemary is widely used in cosmetics, food and medicine industry (Bubonja-Sonje et al., 2011; Tai et al., 2012; Bensebia and Allia, 2016).

Clary sage (*Salvia sclarea*) or clear eye is native herb in Mediterranean area, southern Europe and central Asia (Simon et al., 1984). It is used in traditional medicine for stomach ache, diarrhoea, sore throat swelling and headaches. Furthermore, clary sage has important secondary metabolites which are help to immune responses in animal. Previous studies reported that clary sage has an antioxidant activity, anti-microbial and anti-fungal activity *in vitro* (Dweck, 2000; Pitakorili, 2002; Gülçin et al., 2004; Kuzma et al., 2007).

Thyme (*Thymus capitatus*) is a native herb species in Mediterranean Europe. It has various biological properties such as antioxidant, anti-inflammatory, antiparasitic, anti-bacterial, antitussive and carminative. In addition, some antioxidant components such as carvacrol, thymol are contained in thyme exracts (Mkaddem et al., 2011; Giarratana et al., 2014).

The purpose of this study was tested antiparasitic effects of thyme (*Thymus* sp.), rosemary (*Rosmarinus officinalis*) and clary sage (*Salvia sclarea*) against parasites in common carp.

Material and methods

Identification of Parasites in Common carp (Cyprinus carpio)

The adult (100-310 g) and juvenile common carps (15-20 g) were obtained from the Yoshida Research Station, Tokyo University of Marine Science and Technology, Shizuoka, Japan. Fish naturally infected with parasites were transferred to an indoor laboratory and acclimated in four plastic tanks (200 l) with constant flow of filtered new water (40% water change per 1 h), at > 70% O_2 saturation, 0.2–0.5 mg/L ammonium, 0.02–0.04 mg/L nitrite, pH 7.2 and temperature at 21–22°C. Until the experiments started, fish were fed commercial feed (Feed One Co. LTD., Kanagawa, Japan) at a rate of 1% of body weight per day. Fish were killed by pithing methods for parasite examination. Fish skin, gills and fins were observed for parasite infestation under a light microscope (Olympus CX22LED) and a dissecting microscope (Leica WILD M8). Some parasites were isolated from fresh gills and skin and then prepared for morphological analysis and identification. The parasites were identified as *Ichthyophthirius multifiliis* (tomont stage), *Trichodina* spp., *D. minutus*, *D. extensus* and *Argulus foliaceus* using the criteria proposed by Allison and Rogers (1970), Fernando et al., (1972), Ogawa and Egusa (1977), Gussev (1985) and Woo (2006) (Figure 2.1; Figure 2.2; Figure 2.3; Figure 2.4).

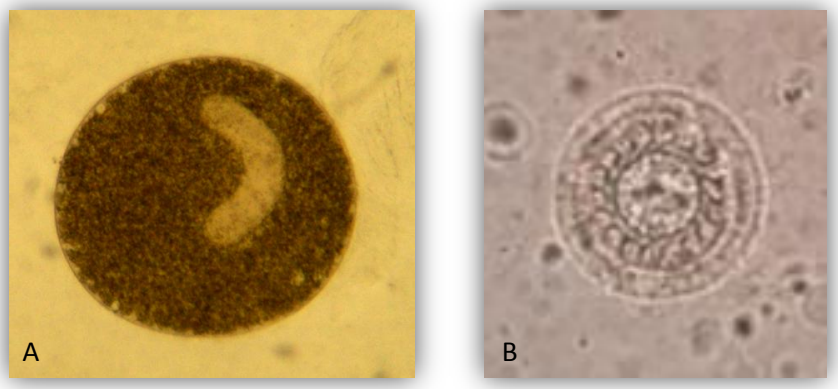

Fig. 2.1. *Ichthyophthirius multifiliis* (tomont stage) (A); *Trichodina* spp. (B).

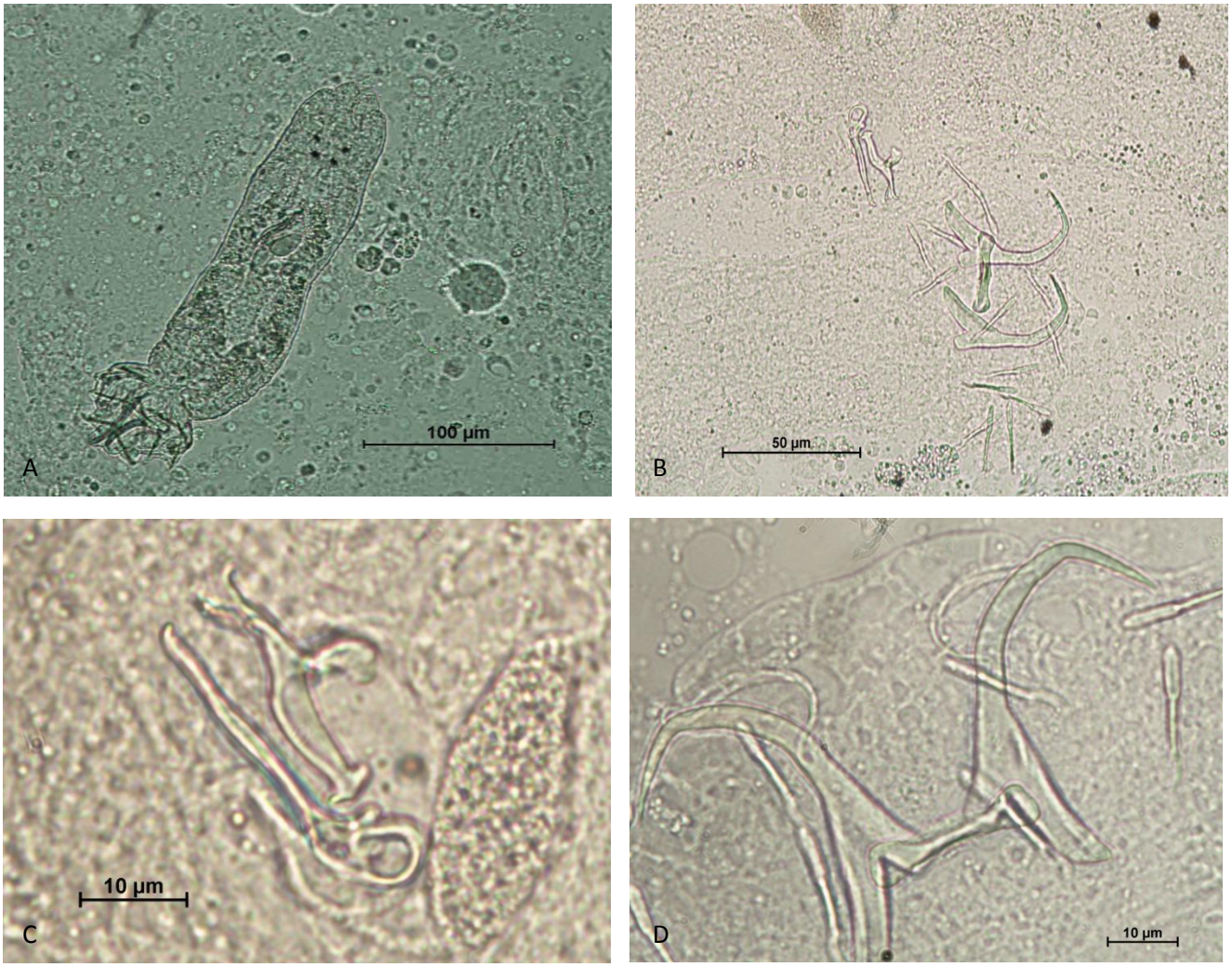

Fig. 2.2 Body morphology of *Dactylogyrus minutus* (A); copulatory organ and hooks of *D. minutus* (B); copulatory organ of *D. minutus* (C); hooks of *D. minutus* (D).

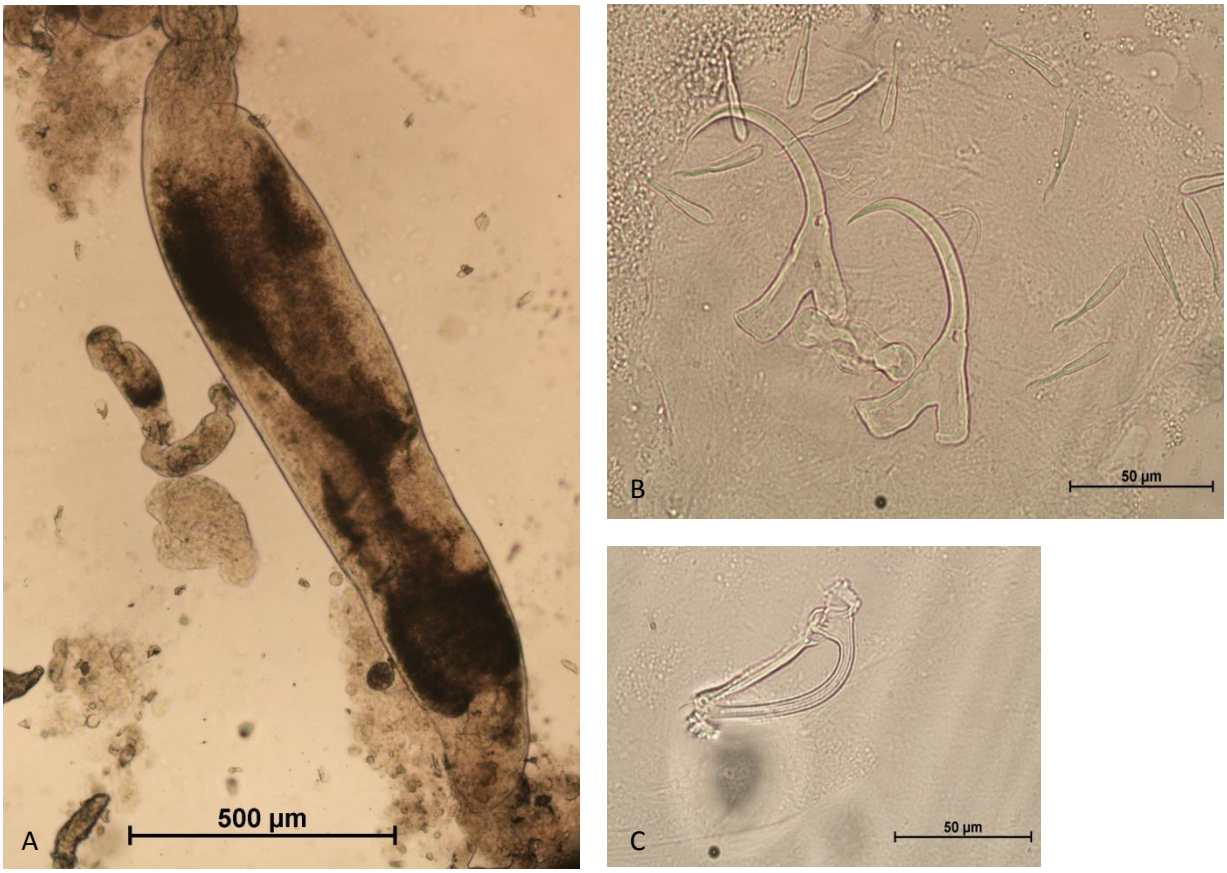

Fig. 2.3. Body morphology of *Dactylogyrus extensus* (A); hooks of *D. extensus* (B); copulatory organ of *D. extensus* (C).

Fig. 2.4. Body morphology of *Argulus foliaceus*.

Preparation of rosemary (Rosmarinus officinalis), clary sage (Slavia sclarea) and thyme (Thymus capitatus) extracts

Leaves of rosemary, clary sage and thyme were purchased in the Tsukiji market, Tokyo, Japan in June of 2015 and kept at 4°C until use. Aqueous extraction (Aq-E) and ethanol extraction (Et-E) were used. Ten grams of leaves and 50 ml of distilled water or 75% ethanol were blended in a laboratory blender at room temperature, forming a fine suspension. The suspensions were transferred to 50 ml plastic tubes, stored overnight at 4°C and centrifuged at 1800 x g for 20 min at 4°C. The supernatants were gently collected by pipet to avoid disturbing the pellet and filtered through 150 μm filter paper. The filtrates were transferred to new plastic tubes and stored at −20°C. The weight of 1 ml of Aq-E and Et-E were determined to be 1 g and 0.8 g respectively.

Antiparasitic effects of rosemary, clary sage and thyme extract in vitro

At least 20 parasites (*Trichodina* spp., *Argulus* spp., *D. extensus* and *D. minutus*) were collected with fine needles and micro pipet from the gills or skin of naturally infected carp and placed into each 12-well culture plates with two replicates per treatment. Aq-E was diluted with distilled water to final concentrations of 100, 150 and 200 g/L. Et-E was diluted with distilled water to final concentrations of 5, 10, 20 and 40 g/L. The parasites were exposed to each herbs extract and observed under a light microscope. Death was judged by cessation or lack of movement and presence of obvious autolysis (Reimschuessel et al., 2011; Zhang et al., 2014; Levy et al., 2015).

Results

Lack of movement and presence of obvious autolysis in all parasite species (*Trichodina* spp., *Argulus foliaceus*, *D. extensus* and *D. minutus*) were examined after addition of each extract concentration within a specific time range.

The range of *Trichodina* spp. survival in water was calculated to be 22 ± 4.2 min in the control groups. All concentrations of rosemary, clary sage and thyme were shown antiparasitic activity against *Trichodina* spp. at 30 min (Table 2.1; Table 2.2; Table 2.3). Rosemary and clary sage extracts had stronger effects than thyme extracts.

Table 2.1
In vitro effects of different rosemary extract concentrations on the survival of *Trichodina* spp. (*n* = 1 replicate; 20 parasites).

In vitro experiments	Dose of extract (g/L)	mortality (%) at 30 min	Time to Death (min)
Water control	0	0	22 ± 4.2
Aq-E	100	100	0
	150	100	0
	200	100	0
Ethanol control	5	100	0
Et-E	5	100	0

Table 2.2
In vitro effects of different clary sage extract concentrations on the survival of *Trichodina* spp. (*n* = 1 replicate; 20 parasites).

In vitro experiments	Dose of extract (g/L)	mortality (%) at 30 min	Time to Death (min)
Water control	0	0	22 ± 4.2
Aq-E	100	100	1.1 ± 0.7
	150	100	0
	200	100	0
Ethanol control	5	100	0
Et-E	5	100	0

Table 2.3
In vitro effects of different thyme extract concentrations on the survival of *Trichodina* spp. (*n* = 1 replicate; 20 parasites).

In vitro experiments	Dose of extract (g/L)	mortality (%) at 30 min	Time to Death (min)
Water control	0	0	22 ± 4.2
Aq-E	100	100	11.2 ± 3.4
	150	100	2.4 ± 0.5
	200	100	0
Ethanol control	5	100	0
Et-E	5	100	0

The survival time of *D. extensus* and *D. minutus* in water was calculated 1012 ± 84.4 min in control groups. The concentration of 100, 150 and 200 g/L Aq-E resulted in parasite's death after 52.2 ± 6.1, 35.2 ± 3.2, 6.4 ± 0.9 min. respectively. The concentration of 5, 10, 20, 40 g/L Et-E killed the parasite after 26.2 ± 4.9, 7.6 ± 1.3, 2.4 ± 0.5, 0 min respectively (Table 4).

The concentration of 100, 150 and 200 g/L clary sage Aq-E resulted in parasite's death after 237.8 ± 18.1, 201.4 ± 10.8, 112.8 ± 17.1 min respectively. The concentration of 5, 10, 20, 40 g/L clary sage Et-E extract killed the parasite after 409.6 ± 53.3, 285.8 ± 48.2, 269.6 ± 50.9, 16.8 ± 4.4 min respectively (Table 5).

The concentration of 100, 150 and 200 g/L thyme Aq-E resulted in parasite's death after 446.5 ± 31.2, 295 ± 47, 226 ± 4.5 min respectively. The concentration of 5, 10, 20, 40 g/L thyme Et-E extract killed the parasite after 311 ± 19.1, 258.5 ± 55.3, 241 ± 14.5, 187.5 ± 10.5 min respectively (Table 6).

Table 2.4
In vitro effects of different rosemary extract concentrations on the survival of *D. extensus* and *D. minutus* (*n* = 1 replicate; 20 parasites).

In vitro experiments	Dose of extract (g/L)	mortality (%) at 1 h	Time to Death (min)
Water control	0	0	1012 ± 84.4
Aq-E	100	100	52.2 ± 6.1
	150	100	35.2 ± 3.2
	200	100	6.4 ± 0.9
Ethanol control	5	0	402.2 ± 57.2
	10	0	310.7 ± 76.5
	20	100	7.2 ± 2.4
	40	100	0 ± 0
Et-E	5	100	26.2 ± 4.9
	10	100	7.6 ± 1.3
	20	100	2.4 ± 0.5
	40	100	0 ± 0

Table 2.5
In vitro effects of different clary sage extract concentrations on the survival of *D. extensus* and *D. minutus* (*n* = 1 replicate; 20 parasites).

In vitro experiments	Dose of extract (g/L)	mortality (%) at 1 h	Time to Death (min)
Water control	0	0	1012 ± 84.4
Aq-E	100	0	237.8 ± 18.1
	150	0	201.4 ± 10.8
	200	0	112.8 ± 17.1
Ethanol control	5	0	402.2 ± 57.2
	10	0	310.7 ± 76.5
	20	100	7.2 ± 2.4
	40	100	0 ± 0
Et-E	5	0	409.6 ± 53.3
	10	0	285.8 ± 48.2
	20	0	269.6 ± 50.9
	40	100	16.8 ± 4.4

Table 2.6
In vitro effects of different thyme extract concentrations on the survival of *D. extensus* and *D. minutus* (*n* = 1 replicate; 20 parasites).

In vitro experiments	Dose of extract (g/L)	mortality (%) at 1 h	Time to Death (min)
Water control	0	0	1012 ± 84.4
Aq-E	100	0	446.5 ± 31.2
	150	0	295 ± 47
	200	0	226 ± 4.5
Ethanol control	5	0	402.2 ± 57.2
	10	0	310.7 ± 76.5
	20	100	7.2 ± 2.4
	40	100	0 ± 0
Et-E	5	0	311 ± 19.1
	10	0	258.5 ± 55.3
	20	0	241 ± 14.5
	40	0	187.5 ± 10.5

The survival time of *A. foliaceus* was recorded 4320-5760 min in the control groups. In addition, rosemary, clary sage and thyme extracts didn't show any antiparasitic effects against *A. foliaceus*.

Discussion

Parasitic infections in fish culture are obstacles in the sustainability and development of the aquaculture industry. Nowadays, synthetic drugs are widely used against parasites. However, these drugs have been reported harmful effects on environment and fish. Therefore, medicinal herb treatment is alternatives treatment material and, they could be show antiparasitic activity against parasites (Levy et al., 2015).

In vitro trials clearly demonstrate the efficacy of rosemary, clary sage and thyme extracts against *Trichodina* spp. . Furthermore, rosemary extracts had an anthelmintic effect against the *D. minutus* and *D. extensus*. During the *in vitro* application of extracts at different

concentrations, Trichodina spp., and monogenean showed their bodycover destroyed or lack of movement and presence of obvious autolysis. Conversely, candidate herbs didn't show antiparasitic effects against *A. foliaceus*. They didn't show any death symptoms *in vitro* applications. *Argulus* spp. is a metazoan parasites, it has a complex body cover called is carapace. For this reason, extracts may not be effectice to *A. foliaceus*. Efficacy of herb extracts probably depends on parasite morphology and their body structures (Woo, 2006).

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Section II

Anthelmintic activity of rosemary (*Rosmarinus officinalis*) against *Dactylogyrus minutus* (Monogenea) infections in *Cyprinus carpio*

Abstract

Monogenean parasites are important ectoparasites of fish, and are responsible for severe economic impacts in the aquaculture industry. They are usually treated with chemicals, but the chemicals can have harmful side effects in the fish and may pose threats to human health. Rosemary (*Rosmarinus officinalis*) is a common medicinal herb, with antimicrobial and antitumor properties. Here, we examined the anthelmintic activity of rosemary extract against the monogenean (*Dactylogyrus minutus*) *in vitro* and *in vivo* using bath treatment and oral administration. The *in vitro* experiments showed that parasite survival was affected by both rosemary extract concentration and the solvent (water and ethanol). Parasites were dead at 61.8 ± 5.6 and 7.8 ± 1.4 min when exposed to 100 and 200 g aqueous rosemary extract solution/L of water respectively. It took 166.7 ± 48.2 and 5.4 ± 1.01 min to kill the parasites when exposed to 1 and 32 g ethanol rosemary extract solution/L of water respectively. Moreover, pure component of rosemary extract obtained commercially used in *in vitro* experiments showed that 1,8-Cineole was the most toxic component of the main components tested. Parasite intensity and prevalence in fish exposed to 50 and 100 g aqueous rosemary solution/L water for 30 min were significantly lower than they were in controls ($p < 0.05$). In oral treatment experiments, diets of *Cyprinus carpio* were supplemented with eight different concentrations of aqueous rosemary extract. The intensity of parasites was significantly less in fish fed for 30 days with feed containing 60, 80 and 100 ml aqueous extract/100 g feed than in control ($p < 0.05$).

Together these results indicate that rosemary is a promising candidate for prevention and control of monogenean infection.

Introduction

Rosemary (*Rosmarinus officinalis*) is a plant belonging to the Lamiaceae family that is native to the Mediterranean region. Rosemaryhas been used as a traditional medicine as well as an ingredient in many medicinal products. The herb is rich in antioxidants (Celiktas et al., 2005; Almela et al., 2006; Erkan et al., 2008). Pharmacological studies have shown that rosemary has anti-inflammatory activity, hepatoprotective, antibacterial, antithrombotic, diuretic, antidiabetic, antinociceptive, anticancer and antioxidant activity (Baratta et al., 1998; Bicchi et al., 2000; Pintore et al., 2002; Linares et al., 2011; Petiwala and Johnson, 2015; Takamaya et al., 2016). Here, I examined the effects of rosemary leaf extracts on *Dactylogyrus minutus* (Monogenea) infection and its possible use as therapeutic agent in aquaculture.

Material and methods

Source of fish and parasite

Source of fish and parasites Common carp (19 ± 0.7 g) were obtained from the Yoshida Research Station, Tokyo University of Marine Science and Technology, Japan. Fish naturally infected with parasites were transferred to an indoor laboratory and acclimated in four plastic tanks (600 l) (300 fish per tank) with constant flow of filtered new water (40% water change per 1 h), at > 70% O_2 saturation, 0.2–0.5 mg/L ammonium, 0.02–0.04 mg/L nitrite, pH 7.2 and temperature at 21–22°C. Until the experiments started, fish were fed commercial feed

(Feed One Co. LTD., Kanagawa, Japan) at a rate of 1% of body weight day^{-1}. Fish skin, gills and fins were observed for parasite infestation under a light microscope (Olympus CX22LED) and a dissecting microscope (Leica WILD M8). *Dactylogyrus* sp. were isolated from the fresh gills and then prepared for morphological analysis and identification. The parasites were identified as *D. minutus* using the criteria proposed by Allison and Rogers (1970) and Ogawa and Egusa (1977). Before and after immersion and oral treatment experiments, adult parasites were counted on host gills to calculate parasite intensity (the number of parasite on the gills) and prevalence (percent of fish that have at least one parasite on the gills) in all treated and control fish as described by Bush et al. (1997).

Preparation of rosemary extracts

Leaves of R. officinalis were purchased in the Tsukiji market, Tokyo, Japan in June of 2015 and kept at 4°C until use. Aqueous extraction (Aq-E) and ethanol extraction (Et-E) were used. Ten grams of leaves and 50 ml of distilled water or 75% ethanol were blended in a laboratory blender at room temperature, forming a fine suspension. The suspensions were transferred to 50 ml plastic tubes, stored overnight at 4°C and centrifuged at 1800 x g for 20 min at 4°C. The supernatants were gently collected by pipet to avoid disturbing the pellet and filtered through 150 μm filter paper. The filtrates were transferred to new plastic tubes and stored at −20°C. The weight of 1 ml of Aq-E and Et-E were determined to be 1 g and 0.8 g respectively.

Determination of five major components of rosemary extracts

Five major components (1,8-Cineole, α-Pinene, β-Pinene, Camphor and Camphene) contained in rosemary extract were analyzed by gas chromatography and mass spectrometry (GC–MS). Distilled water (150 ml) and 4 ml of heptane were added to 2–4 g of the Aq-E sample and distilled by distillation apparatus for 90 min. The heptane fraction (x20 dilution) was used for

GC–MS analysis. Et-E (0.1–0.2 g) was transferred to a 50 ml volumetric flask and a constant volume was maintained by filling the volumetric flask with ethanol to the marked line. The Aq-E sample was analyzed by GC–MS using 7890B/5977A gas chromatographs (Agilent Technologies Inc., Santa Clara, CA, USA) while 6890/5973N gas chromatograph was utilized for the Et-E sample. Separations in the GC were performed on a DB-WAX capillary column (60 m length x 0.25 mm internal diameter x 0.5 μm film thickness; Agilent Technologies Inc.), using helium as the carrier gas (1.0 ml/min). The GC temperature was programmed to increase from 40°C to 220°C at a rate of 10°C/min. MS conditions were: detector interface temperature, 250°C; ion source temperature, 230°C; ionization energy voltage, 70 eV.

Anthelmintic effects of rosemary extract in vitro

Effect of rosemary extracts

Forty *D. minutus* were collected from the gills of naturally infected fish and placed into each 12-well culture plates with two replicates per treatment. Aq-E was diluted with distilled water to final concentrations of 100, 150 and 200 g/L. Et-E was diluted with distilled water to final concentrations of 1, 2, 3, 4, 8, 16 and 32 g/L. The amount of 5 major components in Aq-E and Et-E were calculated based on data from GC–MS analysis. As control, 75% ethanol was diluted with distilled water to final concentration of 1, 2, 3, 4, 8, 16 and 32 g/L were used. The parasites were exposed to each rosemary extract and observed under a light microscope. Death was judged by cessation or lack of movement and presence of obvious autolysis (Reimschuessel et al., 2011; Zhang et al., 2014; Levy et al., 2015). The time of death of each parasite was recorded and the percent mortality (the proportion of dead parasites amongst all parasites) at 1 h was calculated.

Effect of rosemary pure components

The five major pure components of rosemary (1,8-Cineole, α-Pinene, (+)-β-Pinene, (−)-β-Pinene, Camphor and Camphene from Sigma-Aldric, St. Louis, MO, USA were utilized as per the same procedures described in 2.4.1. β-Pinene has two structural isomers, and for this reason, (+)-β-Pinene, (−)-β-Pinene were used.

Acute toxicity test and bath treatment with Et-E and Aq-E

For the toxicity test, fish were deemed dead when opercular movement ceased and they fail to respond to mechanical stimulus (Xiao-Feng et al., 2014). A total of 280 *C. carpio* (weighing 19 ± 0.7 g) were used in the acute toxicity test. The experiment was done in duplicate. Ten infected fish were placed in each aerated 10 L glass aquaria together with Aq-E at 1, 10, 50, 100 and 200 g/L and Et-E at 1, 2, 5 and 10 g/L. Control included water only (without Aq-E) as well as water and 75% ethanol at a concentration of 1, 2, 5 and 10 g/L. Water temperature, oxygen, ammonium, nitrite and pH in the aquariums were 22 ± 1°C, 5–6 mg/L, 0.2–0.5 mg/L, 0.02-0.04 mg/L and 7.3, respectively. Mortalities were recorded at 30 and 60 min. After 1 h, surviving fish were transferred to tanks supplied with constant flow (40% water change per 1 h) filtered water and monitored for 1 week. The Trimmed Spearman-Karber method (Hamilton et al., 1977) with 95% confidence intervals was used to determine the LC_{50} and LC_{90} of the rosemary extracts at 30 and 60 min.

A total of 300 *C. carpio* were divided into fifteen groups in the bath treatment experiment (Table 5). For each group, 10 fish were placed in 10 L glass aerated aquaria (22 ± 1°C) with 2 replicates. Bath treatments lasting 30 and 60 min were assessed for Et-E at concentrations of 0.8 and 1.6 g/L and Aq-E concentrations of 1, 10, 50 and 100 g/L. Fish were exposed to the

treatments for 30 and 60 min and then removed and killed by pithing. At the end of the experiment, the number of gill parasites were counted and parasite intensity and prevalence were calculated (Bush et al., 1997).

Oral treatment trials

Feed preparation

Commercial common carp feed (Feed One Co. LTD., Kanagawa, Japan) was ground in mechanical grinder (Retsch ZM 200, Haan, Germany). Different doses of undiluted Aq-E (5, 10, 15, 20, 40, 60, 80 and 100 ml/100 g feed) were mixed with commercial feed in a horizontal mixer and pelleted into 4.8 mm pellets using a laboratory pellet-making machine (OMC-22B, Omichi, Gunma, Japan). Pellets were dried in a vacuum freeze-drier (RLE-206, Kyowa Vacuum Engineering, Tokyo, Japan) and stored at −20°C until use.

Experimental design

Infected carp (weighing 19 ± 0.7 g) were randomly selected and divided into nine groups consisting of 8 experimental diets 5, 10, 15, 20, 40, 60, 80 and 100 ml Aq-E/100 g of feed and a control diet without any extract. Fifteen fish were placed in each aquarium that had aeration and mechanical filtration. Ammonium, nitrite, temperature and oxygen were monitored every 2 days. Water quality was maintained at 0.2-0.5 mg/L ammonium, 0.02-0.04 mg/L nitrite, 6–7 mg/L of dissolved oxygen during the experiments. Water temperature was kept at 22 ± 1°C. All the groups were fed at a rate of 3% of body weight day^{-1}, and the photoperiod was kept at 12 h. Twenty percent of recirculated water was changed every day. The fish were fed for 30 days. At day 10, 20 and 30, 5 randomly selected fish from each group were examined for parasites as described in the bath treatments.

Statistical analysis

Statistics were analyzed with SPSS software version 24.0 (IBM Corp., Armonk, NY, USA). All results of *in vitro*, bath and oral treatment experiments were compared using one-way ANOVA and multiple comparisons were made using Tukey's multiple range test. Significance was set at $p < 0.05$. Shapiro-Wilk, Barlett, Kruskal-Wallis ($p < 0.05$) and Tukey's test were used to analyze the acute toxicity test data.

Results

Determination of the five major components of R. officinalis from extracts

The compositions of component in each rosemary extract are shown in Table 3.1. 1,8-Cineole (3.1 mg/100 g Aq-E) and Camphor (2.4 mg/100 g Aq-E), were the major components in the aqueous extract, whereas α-Pinene (0.04 mg/100 g Aq-E), β-Pinene (0.03 mg/100 g Aq-E) and Camphene (0.03 mg/100 g Aq-E) were present as minor components. The concentration of 1.8-Cineole (23 mg/100 g Et-E) and Camphor (19 mg/100 g Et-E) in ethanolic extract was more than 7 times that of the aqueous extract, α-Pinene (14 mg/100 g Et-E), Camphene (8.5 mg/100 g Et-E) and β-Pinene (7.2 mg/100 g Et-E) were also found at much higher concentrations than seen in the aqueous extract.

Table 3.1
Major chemical compounds identified from rosemary aqueous and ethanolic extracts and their amount in 100 grams extract.

Component	Molecular formula	Molecular weight	Aq-E (mg/100 g)	Et-E (mg/100 g)
1,8-Cineole	$C_{10}H_{18}O$	154	3.1	23
Camphor	$C_{10}H_{16}O$	152	2.4	19
α-Pinene	$C_{10}H_{16}$	136	0.04	14
Camphene	$C_{10}H_{16}$	136	0.03	8.5
β-Pinene	$C_{10}H_{16}$	136	0.03	7.2

In vitro parasite survival

Effect of rosemary extracts

The cessation or lack of movement and presence of obvious autolysis in *D. minutus* was examined after addition of each extract concentration within a specific time range. The results indicated that all concentrations of ethanol and aqueous extracts containing components 1,8-Cineole, α-Pinene, β-Pinene, Camphor and Camphene produced significant effects on *D. minutus* during *in vitro* experiments. Parasite survival time was dosage-dependent for all extractions. As the Aq-Econcentration increased from 100 to 200 g/L, the mean survival time decreased from about 61.8 ± 5.6 min to about 7.8 ± 1.4 min, while for the water control it was over 1200 min (Table 3.2). As the Et-E concentration increased from 1 to 32 g/l, the mean survival time decreased from about 166.7 ± 48.2 min to about 5.4 ± 1.01 min. Each of these times was several times shorter than for the corresponding ethanol control.

Effect of rosemary pure components

1,8-Cineole had the strongest effect of the components tested (Table 3). Additionally, (−)-β-Pinene showed more significant effects than α-Pinene, (+)-β-Pinene, Camphor and camphene (Table 3.3).

Acute toxicity test and bath treatment with ethanolic and aqueous rosemary extract

No mortality was observed in the control groups. Table 3.4 shows carp mortality after exposure to extracts at different concentrations for 30 and 60 min. The toxicity effect was dependent upon concentration and exposure time. The concentration of 2.1 g/L ethanolic extract was toxic to all fish at 30 min. Body and opercula movement ceased at 30 min. Ethanolic extract at a concentration of 1.2 g/L was toxic to fish at 60 min but not at 30 min.

Aqueous extract at a concentration of 50 g/L caused no mortalities at 30 or 60 min. After 30 min of bath treatment, the intensity of *D. minutus* in gills was significantly lower ($p < 0.05$) with ethanol extract at a concentration of 1.6 g/L than in the water and ethanol control groups and significantly lower ($p < 0.05$) with aqueous extract at concentrations of 50 and 100 g/L than in the water controls (Table 3.5). In the latter case, the rate of prevalence was also lower than in the water controls.

Oral treatment

Aqueous extract in the diet at concentration between 60 and 100 ml/100 g of feed significantly reduced the parasite intensity after 30 days, while lower concentrations had no significant effect (Table 3.6; $p < 0.05$). Indeed, no parasites were found in the 80 and 100 ml/100 g of feed groups after 20 or 30 days.

Table 3.2
In vitro effects of different rosemary extract concentrations on the survival of *Dactylogyrus minutus* (*n* = 2 replicates; 40 parasites per replicate).

In vitro experiments	Dose of extract (g/L)	1,8-Cineole (mg/L)	Camphor (mg/L)	α-Pinene (mg/L)	β-Pinene (mg/L)	Camphene (mg/L)	Mortality (%) at 1 hour	Time to Death (min)	Range of Survival time (min.)
Water control	0	0	0	0	0	0	0	1269 ± 52.4a	982-1366
Aq-E	100	3.1	2.4	0.04	0.03	0.03	85.7	61.8 ± 5.6^{x}e	10-71
	150	4.65	3.6	0.06	0.045	0.045	100	31.4 ± 5.2^{x}e	5-42
	200	6.2	4.8	0.08	0.06	0.06	100	7.8 ± 1.4^{xy}e	1-11
Ethanol control	1	0	0	0	0	0	0	772.8 ± 87.4b	603-873
	2	0	0	0	0	0	0	619 ± 137.5c	315-802
	4	0	0	0	0	0	0	576.6 ± 15.8c	276-599
	8	0	0	0	0	0	0	354 ± 22.6d	109-378
	16	0	0	0	0	0	27.5	161.8 ± 23e	39-201
	32	0	0	0	0	0	100	23.6 ± 2.6e	10-27
Et-E	1	0.23	0.19	0.14	0.085	0.072	0	166.7 ± 48.2^{x}e	103-252
	2	0.46	0.38	0.28	0.17	0.144	68.5	85.45 ± 39.1^{x}e	33-145
	4	0.92	0.76	0.56	0.34	0.288	100	31.4 ± 3.9^{x}e	11-38
	8	1.84	1.52	1.12	0.68	0.576	100	23 ± 2^{x}e	5-26
	16	3.68	3.04	2.24	1.36	1.152	100	10.8 ± 2,4^{xy}f	3-12
	32	7.36	6.08	4.48	2.72	2.304	100	5.4 ± 1,01^{xy}f	1-7

(The amount of components in extract were calculated based on data from experiments shown in Table 1).
Different letters indicate significant differences between treatments ($p < 0.05$).
^{x}Significant difference compared to water and 1, 2, 4, 8 g/L ethanol control.
^{y}Significant difference compared to water and 1, 2, 4, 8, 16 g/L ethanol control.

Table 3.3
In vitro effect of differnet concentrations of five major components on the survival of *Dactylogyrus minutus* (*n* = 2 replicates; 40 parasites per replicate).

In vitro experiments	Concentration (g/L)	Mortality (%) at 1 hour	Time to Death (min)	Range of Survival (min)
Water control	0	0	1269 ± 52.4a	982-1366
1,8-Cineole	0.25	0	191.8 ± 31.5b	96-289
	0.5	57.5	64.6 ± 18.3c	29-92
	1	100	45.2± 7.7c	20-54
	2	100	35.4± 4.3c	13-46
	4	100	32.6 ± 8.6c	11-38
	8	100	0c	0
α-Pinene	4	0	212.2 ± 22.3b	181-237
	8	0	157.4 ± 9.3b	76-178
	16	0	117.8 ± 10.3c	74-134
	32	82.5	64.8 ± 5.5c	31-88
	90	100	29.6 ± 7.9c	23-37
	135	100	22.6 ± 3.9c	19-29
	180	100	8.8 ± 1.4c	3-11
(+)-β-Pinene	4	0	210.2 ± 15.4b	195-227
	8	0	178.6 ± 23.3b	142-202
	16	0	128.4 ± 3.3bc	121-134
	32	0	121 ± 7.8c	110-134
	90	100	44 ± 3.8c	23-52
	135	100	17.4 ± 1.8c	15-19
	180	100	15.6 ± 2c	11-22
(-)-β-Pinene	4	100	49.6 ± 6.5c	20-58
	8	100	15.2 ± 2.5c	7-19
	16	100	13.4 ± 2c	10-17
	32	100	8 ± 1c	3-10
	90	100	1.4 ± 0.5c	1-2
	135	100	0c	0
	180	100	0c	0
Camphor	4	0	412.8 ± 12.3d	363-430
	8	0	369.6 ± 18.3d	306-399
	16	0	294.2 ± 16.4e	276-316
	32	0	234.2 ± 12.1be	192-248
	90	40.3	72.2 ± 11.1c	43-82
	135	86.5	60.8 ± 1.3c	48-66
	180	100	36.8 ± 5.2c	6-49
Camphene	4	0	1292 ± 23af	1002-1370
	8	0	1268 ± 20.2a	1034-1275
	16	0	1341 ± 46.9f	995-1460
	32	0	1267 ± 24.3a	1062-1290
	90	0	1260 ± 38.4a	1022-1365
	135	0	1157 ± 77.6a	980-1401
	180	0	1192 ± 39.3g	892-1302

Different letters indicate significant differences between treatments ($p < 0.05$)

Table 3.4
Acute toxicity test (LC_{50}, LC_{90}) of the rosemary extracts in *Cyprinus carpio* (*n* = 2 replicates; 10 fish per replicate).

Rosemary extract	Time (min)	LC_{50} (g/L)	LC_{90} (g/L)
Ethanol	30	2.1	3.9
	60	1.2	2.4
Aqueous	30	127.2	160.8
	60	92.5	150.1

Table 3.5
Mean intensity (± s.e.m) and prevalence of *Dactylogyrus minutus* infection in *Cyprinus carpio* following bath treatment (*n* = 2 replicates; 10 fish per replicate).

Bath treatment	Concentration (g/L)	Time (min)	Intensity of *D. minutus*	Prevalence of *D. minutus* (%)
Water control	0	60	112.2 ± 32.5a	100
Ethanol (75%) control	0.8	30	130.6 ± 27.87a	100
	1.6	30	104.7 ± 32.2a	100
	0.8	60	113.3 ± 24.5a	100
	1.6	60	101.4 ± 20.4a	100
Rosemary ethanol extract	0.8	30	87.2 ± 16.2a	100
	1.6	30	39.1 ± 27.1b	100
	0.8	60	72.5 ± 15.04b	100
Rosemary aqueous extract	1	30	119.8 ±30.84a	100
	10	30	69.9 ± 19.8ab	100
	50	30	2 ± 3.8b	30
	100	30	0.4 ± 0.8b	25
	1	60	110.6 ± 19.8a	100
	10	60	70.1 ± 30,04b	100
	50	60	2.9 ± 4.09b	30

Different letters indicate significant differences between treatments ($p < 0.05$).

Table 3.6
Mean intensity ± s.e.m of *Dactylogyrus minutus* infection in *Cyprinus carpio* following feeding for 30 d with diet supplemented by rosemary aqueous extract.

Diet experiment group (ml/ 100 g feed)	Intensity of *D. minutus*			
	Day 0	Day 10	Day 20	Day 30
Water control	136.6 ± 30.1a	125.8 ± 28.2a	133.2 ± 25.6a	110.6 ± 16.2a
5	136.6 ± 30.1a	112.8 ± 36.05a	125.4 ± 16.7a	140.2 ± 38.1a
10	136.6 ± 30.1a	136.2 ± 31.5a	111.2 ± 45.8a	129.2 ± 36.5a
15	136.6 ± 30.1a	114.2 ± 16.4a	141.2 ± 54a	100.6 ± 32a
20	136.6 ± 30.1a	108.6 ± 25.3a	95.2 ± 21.04a	73.4 ± 19.8a
40	136.6 ± 30.1a	118.8 ± 29.7a	86.6 ± 20.5 a	49.2 ± 13.4a
60	136.6 ± 30.1a	74.6 ± 11.2a	50.4 ± 26.13b	41 ± 21.3b
80	136.6 ± 30.1a	73.4 ± 52.6a	0 ± 0b	0 ± 0b
100	136.6 ± 30.1a	27 ± 24.5b	0 ± 0b	0 ± 0b

Different letters indicate significant differences between treatments ($p < 0.05$).

Discussion

Monogenean parasites are among the most harmful pathogens in aquaculture systems (Wootten R., 1989; Reverter et al., 2014). They cause stress, reduced growth rates and high mortality in fish stock (Buchmann and Bresciani, 2006; Ogawa, 2015). Chemotherapy has been widely used to prevent parasitic diseases. However, restrictions and prohibitions on the use of chemical bath treatments such as formalin, trichlorfon, praziquantel and hydrogen peroxide, are in place due to their undesired effects on fish health and the environment. This has encouraged the development of efficacious alternatives for parasite treatment (Keimer and Black, 1997; Kennedy, 2007; Reverter et al., 2014). In recent decades, plant extracts have been widely used against parasites (Reverter et al., 2014). Chemotherapy using natural herbs in place of other chemicals has many advantages as a countermeasure in farmed fish from the

perspective of food safety and environmental loading. For example, rosemary has been shown to have an antibacterial effect against streptococcosis infections in tilapia (Abutbul et al., 2004; Zilberg et al., 2010). However, until recently, there has been little research on the anthelmintic effect of rosemary extracts against *D. minutus* (Monogenea). The present study is the first to show that rosemary extracts have significant anthelmintic effects against *D. minutus*.

We have shown that both Aq-E and Et-E have anthelmintic activity, and the activity of Et-E was stronger than that of Aq-E *in vitro*. Ethanolic extract of ginger root (*Zingiber officinale*) (40 g/L for 5 min) eliminated *Gyrodactylus turnbulli* during *in vitro* trials (Levy et al., 2015) and ethanolic extract of Chinese horse chestnut (*Semen aesculi*) (EC_{50} = 5.23 mg/L and EC_{90} = 7.33 mg/L after 48 h) showed anthelmintic activity (Liu et al., 2010). These results compare favorably with our candidate plant, in showing similar anthelmintic activity. However, the active components of these two herbs have not yet been identified.

The major components of rosemary have been reported as 1,8-Cineole, β-Pinene, α-Pinene, Camphor and Camphene by Wang et al. (2008) and Takamaya et al. (2016). The anthelmintic activity of each component was examined *in vitro*. All components except for Camphene showed anthelmintic dose-dependent activity against *D. minutus*. Among the effective components, 1,8-Cineole had the strongest anthelmintic activity and the content of this compound in both extracts was highest. The EC_{50} of 1,8-Cineole against *D. minutus in vitro* was around 0.5 mg/L, corresponding with the concentration included in the same effective dose of ethanolic extract (e.g. 2 g/L) (Table 3.2 and Table 3.3). These results suggest that the anthelmintic activity of the extract is mainly due to 1,8-Cineole. While previous studies have only shown the insecticidal activity of 1,8-Cineole against *Periplenata americana* (American cockroach) and *Culex pipiens molestus* (mosquito) (Scriven and Meloan, 1984; Traboulsi et al., 2002), the present study is the first to demonstrate that it also has anthelmintic effects.

This study, confirms the efficacy of rosemary extracts against a monogenean parasite, and the active component 1,8-Cineole was examined. These findings could lead to the safe and effective application of rosemary extracts as therapeutic agents for parasitic disease in farmed fish.

The immersion method is the standard treatment strategy for prevention and elimination of monogeneans (Reverter et al., 2014). The tolerance level of a fish for toxicity against herbal substances depends upon fish species, condition, age, size, concentration of herb extracts and exposure time (Winkaler et al., 2007; Malherios et al., 2016). To assess the practicality of bath treatment by rosemary extract, we examined the toxicity against the common carp. In the toxicity assays, LC_{50} of Et-E and Aq-E at 30 min exposure were 2.1 g/L and 127.2 g/L, respectively (Table 3.1 and Table 3.4). For 1-h exposure, the LC_{50} was 1.6 g/L for Et-E and 92.5 g/L for Aq-E. In addition, Et-E exposure groups exhibited, some abnormal behavior, such as increased opercular movement, restlessness, jerky movements and swimming upside down. These abnormal behaviors indicate that fish were affected by exposure to ethanol and Et-E (Sambasiva Rao, 1999; Sterling et al., 2016). Conversely, abnormal behaviors were not observed in the Aq-E test groups. Thus, the toxicity of the ethanolic extract is clearly stronger than that of the water extract. It is unclear whether rosemary extract has selective toxic or whether monoterpenes are directly toxicity to fish. The concentration of terpenes in 2.1 g/L (at LC_{50} level for 30 min) of Et-E was calculated as 1.5 mg/L. On the other hand, the concentration of terpenes in 127.2 g/L Aq-E was 7.12 mg/L. Thus, the toxicity of extracts was not correlated with the terpene concentration and it is unlikely that the extract has selective toxicity. The Et-E may possibly contain other unknown toxic substances besides terpenes and the extract should be used judiciously. If Et-E contains other toxic substance, the extracts should be applied with caution. According to our clinical trial results, immersion in 50 g/L

of Aq-E for 30 min was the most effective and safe procedure for bath treatment (Table 5). These conditions also reduced the infection rate. This may be prove to be a practical bath treatment for parasitic infections.

The effectiveness of oral administration of the extract was explored because the extract can be easily incorporated into feed and the oral route eliminates the stresses associated with bath treatments (Kim and Choi, 1998; Hirazawa et al., 2004). For these reasons, we undertook clinical examination of fish following oral administration. Ten days feeding in a dose dependent fashion of experimental fish without any difference in feeding activity compared to control group, parasite intensity significantly decreased (Table 6). Notably, parasites were eliminated completely at doses > 80 ml/100 g of aqueous extract. In addition, no abnormal behaviors were observed in any experimental fish during the clinical trial. Thus, oral administration of rosemary extract is a way to control monogenean parasites. If the active components of rosemary extract in feed are absorbed and reach sufficient concentrations to kill the parasites in host material (plasma and mucus), then parasites die and are then eliminated from fish body by their death (Buchmann and Bresciani, 2006). It is reported that the 1,8-Cineole molecule easily crosses between blood vessels in mice and opossum (Kovar et al., 1987; Mclean et al., 2007). Further investigation in fish on the pharmacokinetics of terpenes, such as 1,8-Cineole, are necessary to confirm the efficacy and safety of oral administration of rosemary extract.

In conclusion, our data demonstrate that rosemary extract has potential as a therapeutic agent against monogenean parasites in fish and the main effective components are terpenes such as 1,8-Cineole and (−)-β-Pinene (Table 3.3). Rosemary extract can be applied by either bath treatment or oral administration. Aqueous rosemary extract demonstrated lower toxicity in the host, and although its anthelmintic activity was slightly inferior to that of the ethanolic

extract, the aqueous extract is a relatively safe and beneficial method for practical control of monogenean parasites.

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Chapter 3

Toxicological effects and pharmacokinetics of rosemary (*Rosmarinus officinalis*) extract in common carp (*Cyprinus carpio*)

Abstract

Rosemary (*Rosmarinus officinalis*) is a new therapeutic candidate against streptococcosis and monogenean infections in fish. However, the toxicity of rosemary and the kinetics of one of its major component (1,8-Cineole) are unclear. In the present study, liver, kidney and intestine histopathology, plasma chemistry, kinetics of 1,8-Cineole in blood and mucus were examined in common carp fed a diet containing rosemary extract. Feeding the fish ≥ 20 ml aqueous extract/100 g feed caused nuclear pyknosis and cell atrophy in the liver. Fish fed ≥ 40 ml aqueous extract/100 g developed pyknotic cells with cytoplasmic vacuoles in the kidney, leading to tubular necrosis. Feeding the fish the rosemary diet for 10 and 20 days increased plasma aspartate aminotransferase levels a sign of liver damage. Following oral administration of 80 ml aqueous extract/100 g feed, the 1,8-Cineole blood level peaked at 60 min at 117.9 ± 3.5 ng/ml. The elimination half-life ($T_{1/2}$) of 1,8-Cineole in the blood was 248 min. In mucus, the levels of 1,8-Cineole at the end of 5, 10 and 20 days were 1.5 ± 0.8 ng, 5 ± 4.1 ng and 6.1 ± 3.8 ng per mg crude dried mucus, respectively. These results suggest that at high doses, rosemary extract diets can cause liver and kidney damage. Further studies are needed to establish a suitable dosage for oral treatment of parasitic diseases.

Introduction

Since 2000, aquaculture has been growing 6% per year and now has become a major food industry in the world market as well as an important source of meat in the human diet. Common carp (*Cyprinus carpio*) is a major cultured fish species, accounting for 10% of

freshwater aquaculture production worldwide. However, it is vulnerable to several bacterial, viral and parasitic diseases (Naylor et al., 2000; Eiras, 2008; FAO, 2016).

Some medicinal herbs have been an effective material for controlling various diseases (Reverter et al., 2014). They can also reduce the risk of diseases and support to animal health. Additionally, herbs have secondary metabolites which have bioactive properties for preventing diseases. Many studies on the application of medical plants for disease control in aquaculture have been carried out. For example, rosemary (*Rosmarinus officinalis*) has been reported to have antibacterial and anthelmintic activities (Pintore et al., 2002; Abutbul et al., 2004; Zilberg et al., 2010; Reverter et al., 2014, Chapter 2). We previous reported that rosemary extract has potential as a therapeutic agent against the monogenean parasite *Dactylogyrus minutus* in fish and that the main effective components are terpenes such as 1,8-Cineole and (-)-β-Pinene. In addition, rosemary extract can be applied by either bath treatment or oral administration. These findings pave the way to using natural herbs as therapeutics against parasitic diseases in farmed fish. In order to use such drugs safety, further studies on their toxicity and kinetics are needed. Here, I describe the toxicological effects and pharmacokinetics of oral administration of rosemary extract in healthy common carp to determine whether it has any adverse effects on fish health.

Material and methods

Rosemary extracts and diet preparation

Leaves of *R. officinalis* were obtained from a store in the Tsukiji market, Tokyo, Japan in April of 2016 and kept at 4^{o}C until use. To prepare aqueous extract (Aq-E), 10 g leaves was homogenized in 50 ml of distilled water in a blender at room temperature, stored overnight at

4 ^{o}C and centrifuged 1800 x g, 4^{o}C for 20 min. The supernatant was gently collected with a plastic pipet, filtered through 150 μm filter paper and stored at -20 ^{o}C.

Commercial common carp feed (Feed One CO. LTD., Kanagawa, Japan) was pulverized in a mechanical grinder (Retsch ZM 200, Haan, Germany). The pulverized feed was mixed with different doses of undiluted Aq-E (10, 20, 40, 80 and 100 ml/ 100 g feed) in a horizontal mixer. The mixtures were pressed into 4.8 mm pellets with a pellet-making machine (OMC-22B, Omichi, Gunma, Japan). The pellets were dried in a vacuum freeze-drier (RLE-206, Kyowa Vacuum Engineering, Tokyo, Japan) and stored at -20^{o}C until use.

Histopathology

A total of 24 common carp (84.1 ± 12.3 g) were obtained from the Yoshida Research Station, Tokyo University of Marine Science and Technology, Japan. The fish were randomly separated into 6 glass aquaria (60 l; 4 fish per tank) with aeration and biological filtration. Water quality was maintained at 0.2-0.5 mg/L ammonium, 0.02-0.04 mg/L nitrite, 6-7 mg/L of dissolved oxygen and pH 7.2. Water temperature was kept at 22 ± 1^{o}C. Photoperiod was 12 h. Ten percent of the recirculated water was replaced with fresh water every day. The five groups (10, 20, 40, 80 and 100 ml Aq-E/100 g of feed) and the control (without extract) were fed at a rate of 3% of body weight once a day for 20 days.

After 20 days, intestine, liver and caudal kidney were collected, fixed in 10% neutral buffered formalin, dehydrated through a series of ethanol solutions (70, 90, 95 and 100%), and embedded in paraffin wax. Five-μm sections were stained with hematoxylin and eosin, mounted on slides and examined under a light microscope (Nikon Eclipse E600, Tokyo, Japan).

Alterations in liver and kidney were evaluated semi-quantitatively by ranking the severity of tissue reactions according to Bernet et al. (1999). The sections were evaluated as no

pathological alteration (0%), low changes (1-20%), mild changes (21-50%), and severe changes (> 50%) pathological alterations.

Clinical biochemistry

Fifteen common carp (84.1 ± 12.3 g) were kept under a 12-hour photoperiod, fed commercial feed (Feed One Co. LTD., Kanagawa, Japan) at a rate of 1% of body weight day^{-1} for 7 days and then randomly placed in three tanks similar to those described above (5 fish per tank). The fish were fed three diets: 0, 10 and 80 ml Aq-E/100 g of feed at a rate of 3% of body weight day^{-1} for 20 days. Water quality parameters were the same as described above. Serum samples were taken at the end of 10 and 20 days. Blood was collected in a 1 ml heparinized syringe with a 26 G x 1/2 needle by puncture of the caudal vein. Whole blood was centrifuged for 8 min, and plasma was collected and stored at -20 ºC until analysis. Blood clinical chemistry analysis was standardized and performed as referred in plasma chemistry analysis operation part.

The plasma samples (200 µl) were analyzed for biochemical parameters in an automated chemical analyzer Hitachi 7020 (Hitachi, Tokyo, Japan) using commercial reagents obtained from Wako (Osaka, Japan). Measured parameters included aspartate aminotransferase (AST) (JSCC transferable method), total cholesterol (TCHO) (Cholesterol oxidase-HMMPS method), triglycerides (TG) (GPO-HMMPS, Glycerol blanking method), creatinine (CRE) (Creatininase-HMMPS method); urea nitrogen (UN) (Urease-GIDH method) and total protein (TP) (Biuret method). All samples were analyzed on the same day for each sampling time.

Pharmacokinetics of 1,8-Cineole

A total of 66 common carp (84.1 ± 12.3 g) were randomly divided into two groups. For each group, 33 fish were placed in 200-l plastic tanks with constant flow of filtered new water (40% water change per 1 h), at > 70% O_2 saturation, 0.2-0.5 mg/L ammonium, 0.02-0.04

mg/L nitrite, pH 7.2 and temperature at 22 ± 1°C. Until the experiments were started, the fish were fed commercial feed (Feed One Co. LTD., Kanagawa, Japan) without rosemary extract diet at a rate of 1% of body weight day^{-1}. The fish were starved for 3 days before administration of the rosemary extract diet. The two groups were fed 0 and 80 ml Aq-E/100 g of feed (3% of body weight), respectively. After oral administration, blood samples (1 ml) were collected from caudal vein puncture with 2.5 ml syringe fitted with a 24 G needle at 0 min, 30 min, 1 h, 2 h, 3 h, 6 h, 12 h, 24 h, 48 h, 72 h and 96 h and then was stored at -20 ºC until GC-MS analysis.

Common carp (84.1 ± 12.3 g) were placed in two tanks (20 fish/tank), and fed 0 and 80 ml Aq-E/100 g of feed) with constant flow of filtered new water (40% water change per 1h), with water conditions similar to those described above. Before the feeding experiment, mucus samples were taken from each experimental group with a plastic sheet. All groups were fed at a rate of 3% of body weight day^{-1} for 20 days. At days 5, 10 and 20, mucus samples were collected from 5 randomly selected fish from each group and stored at -20ºC. Mucus samples were dried in vacuum freeze-drier (RLE-206, Kyowa Vacuum Engineering, Tokyo, Japan). The weight of crude mucus was determined in a microbalance. Ten milligrams of crude mucus were dissolved into 1 ml distilled water and analyzed by GC-MS.

To extract volatile compounds form the fish blood and mucus, 1 ml of whole blood and 10 mg mucus sample were transferred into 10-ml screw cap glass vials. Then a DVB/CAR/PDMS solid phase micro-extraction (SPME) fiber (50/30 μm solid phase thickness; Spelco, MO, USA) was exposed in headspace of the vial for 30 min, with a stirring speed 100 x g at 60 ºC.

1,8-Cineole was detected with a GCMS-2010 gas chromatography system equipped with a QP 2000 mass spectrometer (Shimadzu, Kyoto, Japan) with a DVB/CAR/PDMS solid phase micro-extraction (SPME) fiber (50/30 μm solid phase thickness; Spelco, MO, USA). Volatile

compounds were separated with a Spelcowax-10 capillary column (30 m × 0.25 mm i.d., polyethylene glycol phase thickness of 0.25 μm, Sigma-Aldrich, CA, USA).

The carrier gas was helium with flow rate 1 ml/min. The injector temperature was set at 220ºC. The GC temperature program was: initial temperature of 38ºC, hold for 3 min, increase to 180ºC at 12ºC/min, hold for 5 min, increase to 240ºC, hold for 5 min. The ion source was set at 200ºC and 70 eV. 1,8-Cineole was quantified in selective ion monitoring (SIM) mode with characteristic m/z values of 139 and 154 (Liu et al., 2011).

Statistical analysis

Statistics were analyzed with SPSS software version 24.0 (IBM Corp., Armonk, NY, USA). All results of experiments were compared using one-way ANOVA and multiple comparisons were made using Tukey's multiple range test. Significance was set at $p < 0.05$.

Results

Effects of rosemary extract on histology of liver, kidney and intestine

No abnormal behavior or mortality were observed in any of the fish in the experiments. No histopathological changes were observed in the control. However, fish fed ≥ 20 ml aqueous extract/100 g feed for 20 days showed nuclear pyknosis and cell atrophy in the liver (Fig. 3.1). Necrotic cells and irregular-shaped nuclei were also observed in the liver. Increasing the amount of extract in the feed increased the number of abnormal sized hepatocytes. Fish fed ≥ 40 ml aqueous extract/100 g of feed showed some pathological changes in the kidney, such as pyknotic cells with cytoplasmic vacuoles, leading to tubular necrosis (Fig. 3.2). The intestines did not show any abnormal tissue.

The severity of histological reactions in the liver and kidney increased in a dose-depended manner (Table 4.1).

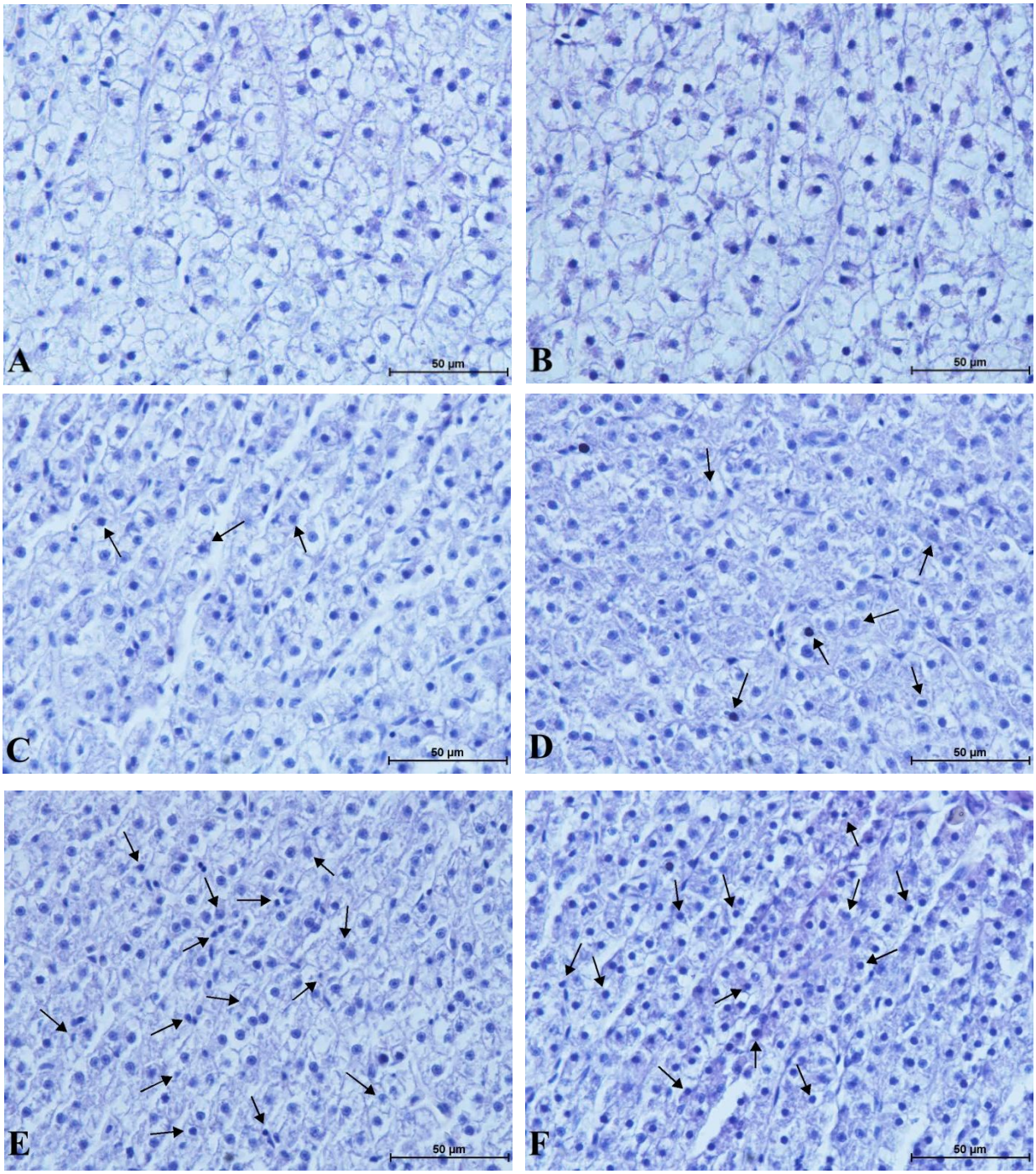

Fig. 3.1 Histopathology of liver of common carp with diet supplemented with rosemary aqueous extract. Diets contained (A) 0, (B) 10, (C) 20, (D) 40, (E) 80 and (F) 100 ml Aq-E/100 g of feed. (A) and (B) show normal hepatocyte structure. (C)-(F) show pyknosis, necrotic cells and irregular shaped nuclei (black arrows) and many of nuclei are condensed, losing their nucleoli. Many of the cells show atrophy.

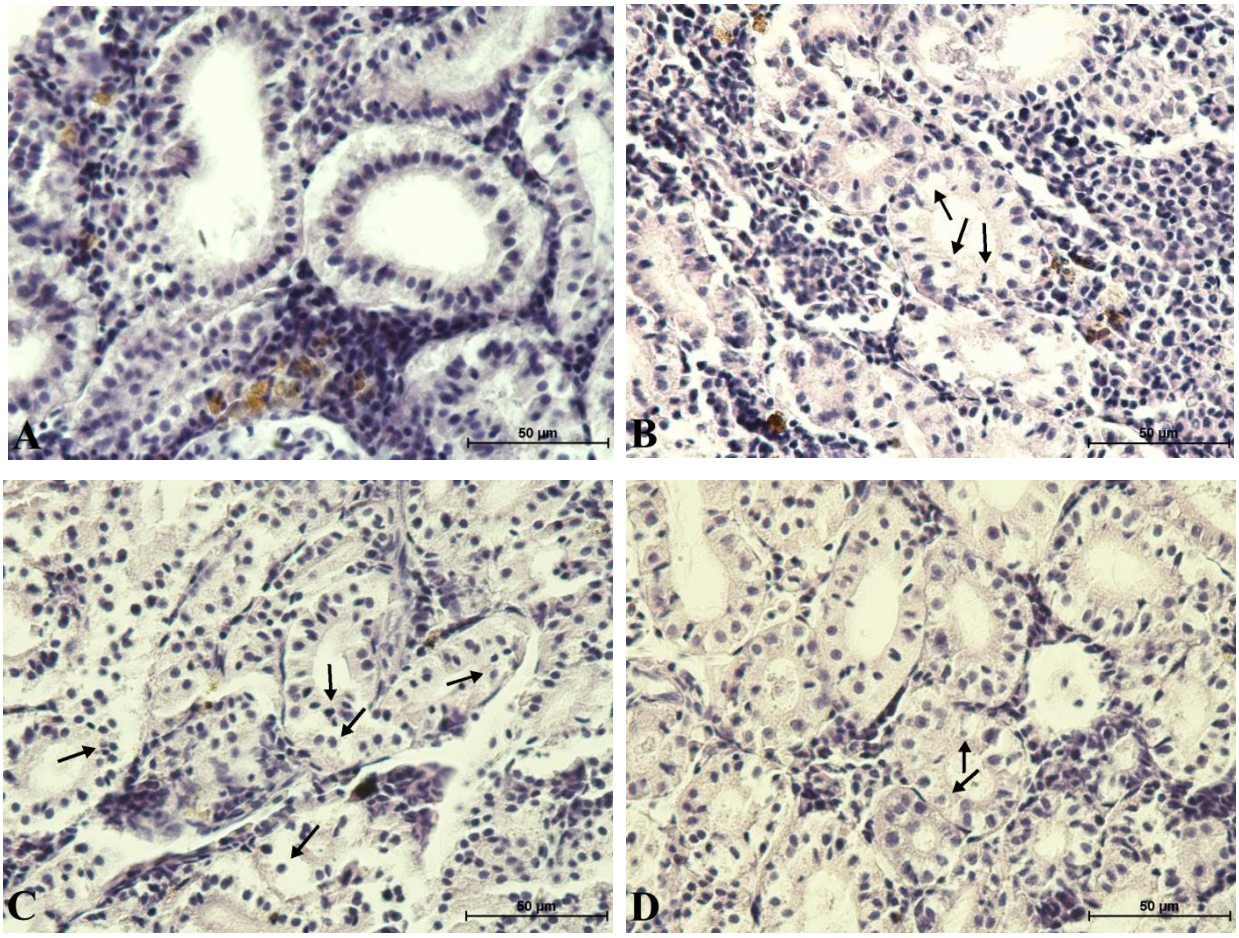

Fig. 3.2 Histopathology of kidney common carp with diet supplemented by rosemary aqueous extract; control (A); 40 ml Aq-E/100 g of feed (B), 80 ml Aq-E/100 g of feed (C) and 100 ml Aq-E/100 g of feed (D) were showed destroyed tubular structure with pyknotic nuclei (black arrow).

Table 4.1
Histopathological changes in liver and kidney of control and rosemary diet groups for 20 d with diet supplemented by rosemary aqueous extract.

Lesion	Control	10 ml Aq-E/100 g of feed	20 ml Aq-E/100 g of feed	40 ml Aq-E/100 g of feed	80 ml Aq-E/100 g of feed	100 ml Aq-E/100 g of feed
Liver						
Pyknotic nucleus	-	-	+ (1/4) ++ (3/4)	++ (4/4)	+++ (4/4)	+++ (4/4)
Cellular atrophy	-	-	+ (1/4) ++ (3/4)	++ (4/4)	+++ (4/4)	+++ (4/4)
Irregular shapes nucleus	-	-	+ (1/4) ++ (3/4)	++ (4/4)	+++ (4/4)	+++ (4/4)
Necrotic cell	-	-	+ (1/4) ++ (3/4)	++ (4/4)	+++ (4/4)	+++ (4/4)
Kidney						
Pyknotic nucleus	-	-	-	+ (1/4) ++ (3/4)	++ (4/4)	++ (4/4)
Cytoplasmic vacuolation	-	-	-	+ (1/4) ++ (3/4)	++ (4/4)	++ (4/4)

-: none (0%), +: low (< 20%), ++: moderate (20-50%) , +++: severe (> 50%) (Cells showing histopathological alterations were counted from approximately 20% of gross area in each section).

1/4: sample of 1 fish, 2/4: sample of 2 fish, 3/4: sample of 3 fish, 4/4: sample of 4 fish showed histopathological alteration.

Clinical biochemistry

No mortality or diminished appetite was observed in any of the experimental groups after 20 days. Aspartate aminotransferase (AST) activity and triglycerides (TG), which when elevated are indications of liver damage, increased slightly but not significantly after 10 and 20 days of rosemary extract feeding (Table 4.2). The number of specimens showing elevated AST activity increased in a dose-dependent manner. No significant changes in other clinical biochemical parameters were observed among the experimental groups.

Table 4.2
Plasma chemistry analysis shown as mean ± SD for each parameter for 20 d with diet supplemented by rosemary aqueous extract.

Diet experiment group	AST (mg/dL)	UN (mg/dL)	TCHO (mg/dL)	TG (mg/dL)	TP (mg/dL)	CRE (mg/dL)
			Day 10			
Control	72	3.9	202.9	464	2.8	0.51
	1	6.1	140.7	440	2.67	0.58
	85	5.5	144.1	325	2.13	0.65
	48	5.8	156.4	423	2.18	0.53
	1	5.4	115.74	286	2.78	0.55
Average ± SD	68.3 ± 39.71	5.34 ± 0.8	152.01 ± 53.02	387.6 ± 77.5	2.5 ± 0.3	0.5 ± 0.05
10 ml/100 g feed	56	5.3	90.29	303	1.65	0.62
	115	8.6	216.69	510	2.95	0.5
	59	6.6	155.3	495	2.36	0.39
	56	7.6	162.11	442	2.39	0.63
	85	5.9	141.15	601	2.57	066
Average ± SD	74.2 ± 25.85	6.8 ± 1.32	153.1 ± 45.3	470.2 ± 109.6	2.3 ± 0.4	0.56 ± 0.11
80 ml/100 g feed	107	8.6	233.32	803	2.94	0.6
	86	7.2	175.99	482	2.39	0.6
	63	6	171.12	463	2.43	0.4
	177	5.7	190.61	336	2.37	0.45
	60	9.4	194.04	553	2.49	0.31
Average ± SD	98.6 ± 47.7	7.38 ± 1.6	193.01 ± 24.4	527.4 ± 172.8	2.5 ± 0.2	0.4 ± 0.1
			Day 20			
Control	33	5.1	140.9	298	1.63	0.28
	165	5	234.04	431	2.99	0.23
	77	8.2	336.74	528	4.15	0.38
	61	5.5	215.29	458	3.17	0.3
	86	4.5	211.9	351	2.58	0.48
Average ± SD	84.4 ± 49.3	5.66 ± 1.46	227.7 ± 39.5	413.2 ± 90.3	2.9 ± 0.9	0.3 ± 0.09
10 ml/100 g feed	76	6	174.53	372	2.47	0.26
	201	5.1	159.88	163	2.61	0.26
	31	5	205.29	702	2.81	0.61
	177	6.3	255.7	528	2.78	0.47
	86	6	181.48	308	2.25	0.65
Average ± SD	114.2 ± 71.8	5.68 ± 0.5	195.3 ± 37.4	414.6 ± 207.3	2.5 ± 0.2	0.45 ± 0.18
80 ml/100 g feed	271	6.3	187.99	352	3.04	0.33
	179	6.3	290.39	749	3.48	0.43
	41	8.8	290.26	472	3.28	0.5
	146	4.4	241.97	503	3.38	0.31
	81	6.5	243.45	398	3.19	0.61
Average ± SD	143.6 ± 89.3	6.46 ± 1.5	250.8 ± 42.4	494.8 ± 154.08	3.2 ± 0.16	0.4 ± 0.12

Abbreviations - Aspartate aminotransferase (AST), Urea nitrogen (UN), Total cholesterol (TCHO), Triglycerides (TG), Total protein (TP), Creatinine (CRE).

Kinetics of 1,8-Cineole

1,8-Cineole was detected in the blood obtained of fish fed 80 ml Aq-E/100 g feed for 1 day. The blood 1,8-Cineole level peaked at 117.89 ± 3.47 ng/ml at 60 min after oral administration and then decreased exponentially until 720 min (Fig. 3.3). The elimination half-life ($T_{1/2}$) of 1,8-Cineole in the blood was calculated at 248 min.

The concentrations of 1,8-Cineole in crude dried mucus after 5, 10 and 20 days were 1.5 ± 7.6, 5.0 ± 4.1 and 6.1 ± 3.8 ng per mg, respectively (Table 4.3).

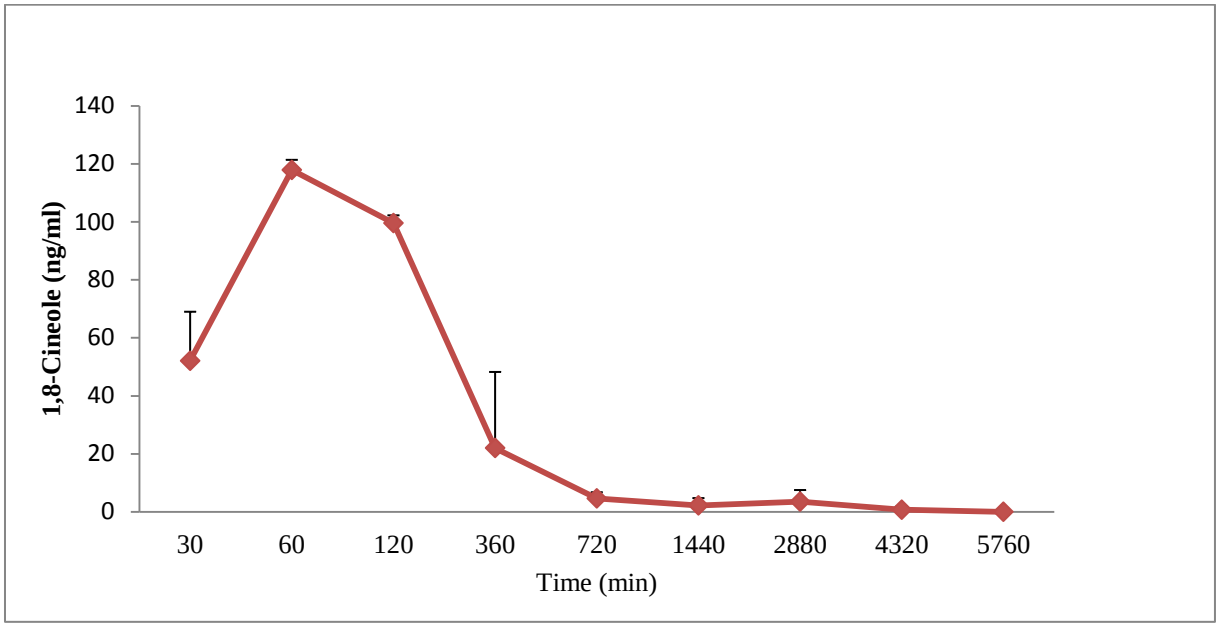

Fig. 3.3 Blood of 1,8-Cineole level in common carp after oral administration of 80 ml aqueous extract/100 g feed (mean ± S.E.M.).

Table 4.3
Mucus level of 1,8-Cineole in fish following feeding for 20 d with diet supplemented by rosemary aqueous extract.

Diet experiment group (ml/ 100 g feed)	1,8-Cineole in mucus (ng/ 10 mg)			
	Day 0	Day 5	Day 10	Day 20
Water control	0	0	0	0
80	0	15.08 ± 7.6	49.8 ± 41.1	60.9 ± 37.7

Discussion

Because of concerns about the impact of aquaculture drugs on the environment and food safety, there has been growing interest in the use of natural herbs as therapeutics for farmed fish. Rosemary has been used to prevent streptococcosis and monogenean parasite infections in fish (Abutbul et al., 2004; Zilberg et al., 2010, Chapter 2). However, little is known about its toxicity or kinetics in healthy fish. Hence, this study was designed to determine the safety of rosemary extract against fish, and obtain some knowledge for establishing practical therapy by the herb.

The present results showed that feeding rosemary Aq-E for 20 days caused some dose-dependent histopathological alterations in the liver and kidney. Therefore, it is concluded that rosemary extract has hepatotoxicity and nephrotoxicity against fish and the lowest observed adverse effect level (LOAEL) is 20 ml Aq-E/100 g feed based on the hepatotoxicity results. Rosemary extracts contain monoterpenes (e.g., 1,8-Cineole, Camphor) and sesquiterpenes (e.g., Caryophyllene) (Takamaya et al., 2016). High doses of monoterpenes, sesquiterpenes and other terpenes are hepatotoxic in human and mice (Zárybnický et al., 2017). For example, camphor causes serious liver laceration and necrotic changes in the central nervous system (Zárybnický et al., 2017). Additionally, 1,8-Cineole has been reported to cause loss of consciousness, depression of reflexes and serious poisoning in human (doses of < 1g/kg) and

rat (LD_{50} = 2.5 g/kg) (Dziba et al., 2006; Mclean et al., 2007; Zárybnický et al., 2017). A diet of 1,8-Cineole (192.45 mg/kg/day) causes some serious pathological changes in kidney such as vacuolar degeneration in mice (Xu et al., 2014). In the present study, the dose of 1,8-Cineole was calculated as 0.186 mg/kg BW/day based on the proximate composition of 1,8-Cineole in Aq-E (3.1 mg/100g Aq-E) (Chapter 2). The dose of 1,8-Cineole in the present study is quite low in comparison with the toxic level in mammals. Therefore, it is considered that the hepatotoxicity and nephrotoxicity of rosemary extract observed in the present study were not caused by monoterpenes but by other toxic components in the extract. A diet containing rosemary extract should be used cautiosly, and before oral administration it is necessary to determine the safe doses.

AST in plasma is an indicator of hepatocyte damage (Hoffman and Solter, 2008). When the liver parenchymal cells or membrane permeability are damaged, the level of AST enzymes increases in the blood (Bhat et al., 2017). AST activity increased slightly but not significantly after 10 and 20 days in the rosemary diet groups. The number of fish that showed higher AST activity increased in a dose-dependent manner, in agreement with histopathological observations in the liver. Because other clinical biochemical parameters were not significantly different among the experimental groups, these results suggest that the liver function (indicated by a decrease of total cholesterol) and kidney damage (indicated by increases of urea nitrogen and creatinine) were not serious. No abnormal behaviors, such as a decrease of appetite, were observed in any experimental fish during the experiments. The clinical biochemistry and behavioral observations suggest that oral administration of rosemary extract does not cause severe disorders in liver or kidney function, even though it caused some histopathological alterations. In our previous study, parasites were completely eliminated at doses > 80 ml/100 g diet of Aq-E for 10 days (Chapter 2). Although we observed no disorders in liver or kidney function, the LOAEL of rosemary extract estimated from the

histopathological examinations in the present study was lower than the effective dose for oral treatment. Further studies are needed to determine the most effective and safe dosage for anthelmintic treatment.

In the present study, the kinetics of rosemary extract was examined by using the concentration of 1,8-Cineole as an index. The blood 1,8-Cineole level peaked at 60 min after administration, suggesting that 1,8-Cineole contained in rosemary extract is quickly absorbed from the digestive tract and transferred to the blood. When herbs that contain 1,8-Cineole are eaten by mammals (human, mice and opossum), they can be easily detected in the blood by their 1,8-Cineole content. Several studies detected 1,8-Cineole in human blood after the termination of rosemary oil inhalation (Kovar et al., 1987; Jäger et al., 1996; Mclean et al., 2007; Horst and Rychlik, 2010). Our results show that after oral administration of rosemary extract, the concentration of 1,8-Cineole rapidly increased in the blood and was distributed to the organs. In addition, the elimination half-life of 1,8-Cineole (248 min) suggests that the absorbed 1,8-Cineole is eliminated quickly.

The mucus layer is the first line of defense against pathogens (Subramanian, 2007; Guardiola, 2014). Dietary factors play a key role in the mucus defense against pathogens (Esteban, 2012). We detected 1,8-Cineole in fish mucus during the 20 days feed with diet of 80 ml/100 Aq-E. We also showed that 1,8-Cineole in rosemary extract is transferred to the mucus in fish by oral administration. Some ectoparasites including the monogenean *Dactylogyrus minutus* consumes fish mucus as a nutrient (Buchmann and Bresciani, 2006). These results suggest that administration of rosemary extract would be effective against those ectoparasites. The concentration of 1,8-Cineole in mucus increased with the administration period. Our results suggest that 1,8-Cineole in mucus would reach an antiparasitic concentration by repetitive administration. Adding myrtle (*Myrtus communis*) to the diet of zebrafish was found to alter

immune gene expression in skin mucus (Safari et al. 2017). Since myrtle contains 1,8-Cineole, 1,8-Cineole may have an effect on the mucus immune system in common carp.

In conclusion, our results show that substances other than monoterpenes in rosemary aqueous extract are hepatotoxic and nephrotoxic, although they did not appear to interfere with liver or kidney function. Further studies are needed to confirm that the histopathological alterations in liver and kidney recover after the termination of rosemary extract administration. Such studies will help establish a suitable dosage of the herb for oral treatment of parasitic diseases.

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Chapter 4

General Discussion

This study was divided into two parts (Chapter 2 and Chapter 3). In chapter 2, I selected three candidate herbs (rosemary, clary sage, thyme) for target of my study and their antiparasitic activity against common carp parasites was examined *in vitro*. It was revealed that rosemary was the most effective against parasites amoung the candidate herbs. It was found that rosemary could eliminate parasite (*Trichodina* spp., *Dactylogyrus minutus* and *D. extensus*) completely at low dose. In addition, when comparing between the efficacies of the ethanolic extract was found to be much more effective than the aqueous extract. These findings are the first report on the antiparasitic (to *Trichodina* spp.) and anthelmintic activitiy (to *D. minutus*, *D. extensus*) of rosemary.

The GC-MS analysis of extracts of rosemary identified five major compound: 1,8-Cineole, α-Pinene, β-Pinene, Camphor and Camphene. The five major effective components of rosemary extracts were examined for anthelmintic activity against *D. minustus in vitro*. *In vitro* experiments showed that 1,8-Cineole was the most effective component against *D. minutus*. This result suggest that we can control the dose of rosemary extracts properly by the monitoring of 1,8-Cineole concentration in the extract.

The results ethanolic extract of rosemary showed higher toxicity than the aqueous extract of rosemary in immersion.

Based on the above results, I tried to cure the infected fish with *D. minutus* by the bath treatment. It is revealed that anthelmintic effect of rosemary extract were dose dependent and minimum effective and safety dose of aqueous extract was 50 g/L for 30 min.

In addition, I also tried to cure the infected carps by oral administration for 30 days. The parasites were eliminated completely in high concetration of extract groups (80 and 100 ml of aqueous extract/100 g of feed) at 20 days. I confirmed that the rosemary extract has the

potential as antiparasitic agent both in immersion and oral administration. Oral administration has the great advantage in comparison with bath treatment. Therefore, I conducted some experiments in order to apply rosemary extract for therapy of parasitic disease in fish in the next chapter.

In Chapter 3, I examined toxicity of rosemary extract by oral administration. Some histopathological alterations were observed in the liver and kidney with dose dependent manner. The LOAEL of rosemary extract was ≥ 20 ml aqueous extract/100 g evaluated by the histopathological observation in the liver. However, no abnormal behaviors, decrease of appetite or symptoms in appearance were observed in any experimental fish during the experiments. In addition, results of clinical biochemistry suggested that oral administration of rosemary extract might not cause severe disorder in liver and kidney function against fish even if the histopathological alterations were observed. The LOAEL of rosemary extract were lower than that of effective dose obtained in the former chapter. Further studies are needed in order to apply rosemary extract to therapy of parasitic disease safely.

The other main finding of this study is that during oral administration of rosemary extract, 1,8-Cineole (main active component of rosemary extract) rapidly transferred to the blood. In addition, the kinetics of 1,8-Cineole in fish similar to mammals in that it is removed from blood quickly. Further research in fish is needed to understand that where it is metabolized, what the metabolites are and how the metabolites are excreted.

In the present study, 1,8-Cineole was detected in the skin mucus of the fish fed with rosemary extract supplemented diet. The concentration of 1,8-Cineole in mucus increased and accumulated during administration period. The accumulation of 1,8-Cineole in fish might be effective against ectoparasite or other pathogens (bacteria, virus or fungus) species which are

affected and killed by 1,8-Cineole. In addition, 1,8-Cineole can support and effect on mucus immune system in fish.

List of Publications and Communications

This thesis contains research that has been published and/or presented in the following:

Publish Paper

Zoral, M.A., Futami, K., Endo, M., Maita, M., Katagiri, T., 2017. Anthelmintic activity of *Rosmarinus officinalis* against *Dactylogyrus minutus* (Monogenea) infections in *Cyprinus carpio*. Vet. Parasitol., 247, pp. 1-6.

Under review

Zoral, M.A., Ishikawa, Y., Ohshima, T., Futami, K., Endo, M., Maita, M., Katagiri, T. Toxicological effects and pharmacokinetics of rosemary (*Rosmarinus officinalis*) extract in common carp (*Cyprinus carpio*). Aquaculture

Conference Communications

The anthelmintic effects of rosemary (*Rosmarinus officinalis*) extracts in common carp (*Cyprinus carpio*) and pharmacokinetic activity of 1,8-Cineole component, International Symposium of Fisheries Science for Future Generation, Tokyo, JAPAN (September 22-24, 2017). (Oral Communication)

First report on the anthelmintic activity of rosemary (*Rosmarinus officinalis*) against monogenea (*Dactylogyrus minutus*) in common carp (*Cyprinus carpio*), International Conference on Advances in Fish Health, Kuala Lumpur, Malaysia (April 4-6, 2017). (Oral Communication)