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Original article

In vitro activity of some essential oils alone and in combination against the fish pathogen *Nocardia seriolae*

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Abstract

Microplate resazurin assay was applied to investigate the *in vitro* activity of four essential oils (EOs); cinnamon (*Cinnamomum zeylanicum*), thyme (*Thymus vulgaris*), lemongrass (*Cymbopogon flexuosus*) and tea tree (*Melaleuca alternifolia*) oils against 80 clinical isolates of the fish pathogen *Nocardia seriolae*. The checkerboard test was then used to determine the possible synergistic effect of EOs combination against reference type strains of fish nocardiosis. All tested EOs had antibacterial activity against *N. seriolae* isolates. Among the tested EOs, cinnamon and thyme oils both exhibited the lowest minimum inhibitory concentrations (MICs) with 5-160 and 10-160 µg/ml, respectively. The activities of lemongrass and tea tree EOs were noted to be less effective with MICs of 20-640 and 160->5120 µg/ml, respectively. The checkerboard panel of cinnamon-thyme EOs combination against *N. seriolae ATCC43993* demonstrated a synergistic effect with a fractional inhibitory concentration (FIC) index of 0.75. For *N. salmonicida ATCC27463,* the combination panel showed an additive effect with an FIC index of 1.0. For *N. asteroides ATCC19247*, the combination panel demonstrated an indifference effect with an FIC index of 1.125. These results indicate that thyme and cinnamon oils alone or the combination of them at a given ratio has a promising potent clinical significance in the treatment of fish nocardiosis. Despite the promising results given by our *in vitro* studies, the clinical benefits of these EOs combinations can only be determined through carefully designed *in vivo* experimental studies.

Key words: cinnamon, thyme, essential oils, *Nocardia seriolae*, MIC, synergy

Introduction

The genus *Nocardia* comprises several species that are known to be unusual causes of a wide spectrum of clinical diseases in both humans and animals (Brown and McNeil 2003).Three species of *Nocardia* have been isolated from diseased fish, namely *N. salmonicida, N. seriolae,* and *N. asteroids* (Austin and Austin 2007). Fish nocardiosis due to *N. seriolae* is a common bacterial infection which affects a wide range of cultured marine and freshwater fish species such as Japanese flounder, sea bass, striped mullet,

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yellowtail, tiger fish, large yellow croaker, snakehead spotted butterfish and snubnose pompano in South East Asia (Chen et al. 2000, Miyoshi and Suzuki 2003, Huang et al. 2004, Wang et al. 2005, Wang et al. 2007, Labrie et al. 2008, Wang et al. 2009, Wang et al. 2014, Vu-Khac et al. 2016). In addition, *N. seriolae* has been reported to cause chronic mortalities in cultured weakfish in the USA (Cornwell et al. 2011) and in cultured meagre in Greece (Elkesh et al. 2013). This disease is characterized by the formation of abscesses in the epidermis and of tubercles in gills, kidneys, and spleens (Kumamoto et al. 1985). Despite the significant economic loss due to nocardiosis, there are no commercial prophylactic measures against *N. seriolae* (Shimahara et al. 2010) except for sulfamonomethoxine and sulfisizol that have been permitted as effective drugs for treatment of *N. seriolae* infections in Japan (Ismail et al. 2012).

The overuse of antibiotics and consequent antibiotic selection pressure is thought to be the most important factor contributing to the appearance of different kinds of resistant microbes (Mimica-Dukic et al. 2003). The previously proposed environmental selective pressure for the routinely used oxytetracycline (OTC) and erythromycin (Em) antibiotics in the Asian aquaculture resulted in a marked prevalence of the h-glucosidase (glu)-positive *N. seriolae* that were OTC resistant and Em sensitive in Asian countries, while the h-glu-negative *N. seriolae* that were OTC sensitive and Em resistant prevailed in Japan (Ismail et al. 2011a, 2011b). For these reasons, the search for antimicrobial safer compounds that have no harmful effects on fish, consumers and the environment is greatly encouraged.

Essential oils (EOs) are complex mixtures of volatile secondary metabolites from plants and they are used to control various infectious diseases (Moghaddasi 2010). Many essential oils are known for their antimicrobial properties and are considered the most widely used natural product to date (Nakatsu et al. 2000). The use of herbal extracts is widely expected to become an alternative therapy in aquaculture as a prophylactic and to control fish diseases. Currently herbs play a considerable role in aquaculture. Several studies have reported that herbal extracts have potential as antimicrobials against various fish pathogens (Rattanachaikunsopon and Phumkhachorn 2010, Alsaid et al. 2010, Talpur and Ikhwanuddin 2012). Thus, it is of interest to study the potential of EOs as antimicrobial agents against *N. seriolae*. The aim of this study is to investigate the *in vitro* activity of four EOs alone and in combination; cinnamon (*Cinnamomum zeylanicum*), thyme (*Thymus vulgaris*), lemongrass (*Cymbopogon flexuosus*) and tea tree (*Melaleuca alternifolia*) oils against fish nocardiosis.

Materials and Methods

Bacterial strains

Eighty *N. seriolae* strains were collected in Japan from the gills, kidneys or spleens of diseased yellowtail (n = 46) and amberjack (n = 34). All strains showed Gram-positive, acid-fast filamentous or branching bacilli under the light microscopy and a positive reaction by species-specific PCR targeting the 16S rRNA gene as previously described (Miyoshi and Suzuki 2003).

Microplate resazurin assay (MRA)

Log-phase cells were used for inoculum preparation equivalent to no. 0.5 McFarland standard as previously reported (Ismail et al. 2011a), and were diluted 100-fold with sterile 0.85% NaCl (10^5 to 10^6 CFU/ml).

Cinnamon (*Cinnamomum zeylanicum*), thyme (*Thymus vulgaris*), lemongrass (*Cymbopogon flexuosus*) and tea tree (*Melaleuca alternifolia*) EOs were obtained from Naturas Psychos (http://www.naturas-psychos.com) as 100% pure oils. Oil suspensions were prepared in sterile distilled water with 5% dimethyl sulfoxide (DMSO) to yield stock solutions. The minimum inhibitory concentrations (MICs) for EOs were determined using the broth microdilution method as previously described (Webster et al. 2010) with some modifications. Serial 2-fold dilutions of the oils in double strength cation-adjusted Mueller Hinton broth (CAMHB; Difco, Michigan) with 5% DMSO were prepared immediately before use. The prepared dilutions were dispensed (100 µl/well) in sterile U-bottom 96-well microtiter plates (Becton Dickinson Lab, New Jersey). To each well, 100 µl of the above-mentioned inoculum was added, resulting in final concentrations from 10^4 to 10^5 CFU/ml. The final concentrations of the oils ranged from 5 to 5120 µg/ml. The micro-plates were covered and mixed for 2min on a Micro mixer (Iwaki Sangyo, Tokyo, Japan) and were incubated at 25°C for 4 days. Each panel contained a control well without oil or inoculum. The type strains *N. seriolae ATCC 43993* and *N. salmonicida ATCC27463* were used as controls for the validation of the identification scheme.

A fresh working solution of resazurin sodium salt powder (Sigma-Aldrich, USA) was prepared at a concentration of 0.01% (w/v) in distilled water and filtered through a 0.2µm membrane (Millipore Corp., New Bedford, MA).

After the microplates were incubated at 25°C for 4 days, 30 µl of the resazurin working solution was

Table 1. Checkerboard panel with the different combination concentrations of thyme (T) and Cinnamon (Cin). Subscribts indicate concentration (µg/ml). C+ve indicates a well containing no oils and C-ve indicates a well containing no bacteria.

	1	2	3	4	5	6	7	8	9	10	11	12
A	**C+ve**	**T_5**	**T_{10}**	**T_{20}**	**T_{40}**	**T_{80}**	**T_{160}**	**T_{320}**	**T_{640}**	**T_{1280}**	**T_{2560}**	**T_{5120}**
B	**Cin_5**	T_5 Cin_5	T_{10} Cin_5	T_{20} Cin_5	T_{40} Cin_5	T_{80} Cin_5	T_{160} Cin_5	T_{320} Cin_5	T_{640} Cin_5	T_{1280} Cin_5	T_{2560} Cin_5	T_{5120} Cin_5
C	**Cin_{10}**	T_5 Cin_{10}	T_{10} Cin_{10}	T_{20} Cin_{10}	T_{40} Cin_{10}	T_{80} Cin_{10}	T_{160} Cin_{10}	T_{320} Cin_{10}	T_{640} Cin_{10}	T_{1280} Cin_{10}	T_{2560} Cin_{10}	T_{5120} Cin_{10}
D	**Cin_{20}**	T_5 Cin_{20}	T_{10} Cin_{20}	T_{20} Cin_{20}	T_{40} Cin_{20}	T_{80} Cin_{20}	T_{160} Cin_{20}	T_{320} Cin_{20}	T_{640} Cin_{20}	T_{1280} Cin_{20}	T_{2560} Cin_{20}	T_{5120} Cin_{20}
E	**Cin_{40}**	T_5 Cin_{40}	T_{10} Cin_{40}	T_{20} Cin_{40}	T_{40} Cin_{40}	T_{80} Cin_{40}	T_{160} Cin_{40}	T_{320} Cin_{40}	T_{640} Cin_{40}	T_{1280} Cin_{40}	T_{2560} Cin_{40}	T_{5420} Cin_{40}
F	**Cin_{80}**	T_5 Cin_{80}	T_{10} Cin_{80}	T_{20} Cin_{80}	T_{40} Cin_{80}	T_{80} Cin_{80}	T_{160} Cin_{80}	T_{320} Cin_{80}	T_{640} Cin_{80}	T_{1280} Cin_{80}	T_{2560} Cin_{80}	T_{5820} Cin_{80}
G	**Cin_{160}**	T_5 Cin_{160}	T_{10} Cin_{160}	T_{20} Cin_{160}	T_{40} Cin_{160}	T_{80} Cin_{160}	T_{160} Cin_{160}	T_{320} Cin_{160}	T_{640} Cin_{160}	T_{1280} Cin_{160}	T_{2560} Cin_{160}	T_{5120} Cin_{160}
H	**Cin_{320}**	T_5 Cin_{320}	T_{10} Cin_{320}	T_{20} Cin_{320}	T_{40} Cin_{320}	T_{80} Cin_{320}	T_{160} Cin_{320}	T_{320} Cin_{320}	T_{640} Cin_{320}	T_{1280} Cin_{320}	T_{2560} Cin_{320}	**C-ve**

added to each well, and the plates were incubated overnight for color development. The MIC was defined as the lowest oil concentration which prevented color change from blue to pink (Palomino et al. 2002).

The checkerboard test

To determine the effect of combinations of EOs at different concentrations, the checkerboard method was used. Reference type strains; *N. seriolae ATCC43993*, *N. salmonicida ATCC27463*, and *N. asteroides ATCC19247* were used as representative strains of fish nocardiosis. The checkerboard assay was performed as previously reported (Dougherty et al. 1977) with minor modifications. Double strength dilutions of oil A and oil B were prepared as previously mentioned, and each of them was dispensed (50 µl/well) in sterile U-bottom 96-well microtiter plates (Becton Dickinson Lab, New Jersey). Each well of oil A/ oil B different combinations (Table 1) was then inoculated with 100 µl of the above-mentioned inoculum resulting in a final concentration of approximately 10^5 CFU/ml, and the microtiter plates were then covered, mixed for 2 min on the Micro mixer (Iwaki Sangyo, Tokyo, Japan) and were incubated at 25°C for 4 days.

After the incubation, the antibacterial activity was detected using the resauzurin colorimetric method by adding 30 µl of resauzurin aqueous solution (0.01% w/v) to each well, and the plates were incubated overnight for color development.

The effects of combinations were evaluated by calculating the fractional inhibitory concentration (FIC) index for the combination using the following formula: FIC of oil A=MIC of oil A in combination/MIC of oil A alone; FIC of oil B=MIC of oil B in combination/MIC of oil B alone; FIC index=FIC of oil A+FIC of oil B (Pillai et al. 2005). Synergy was defined as an FIC index <1. When the FIC index equaled 1.0, it indicated an eaddition f effect. When the FIC index fell between 1.0 and 2.0, it indicated an eindifference f between the agents. An FIC >2.0 would indicate that there was antagonism between the two agents (Pei et al. 2009).

Results

Microplate resazurin assay (MRA)

The MRA results are shown in Fig. 1. Both thyme and cinnamon EOs demonstrated strong activities against *N. seriolae* with MICs of 5-160 and 10-160 µg/ml, respectively. The two most frequent MICs for both thyme and cinnamon EOs were 20 and 40 µg/ml. The activities of lemongrass and tea tree EOs were noted to be less effective with MICs of 20-640 and 160->5120 µg/ml, respectively.

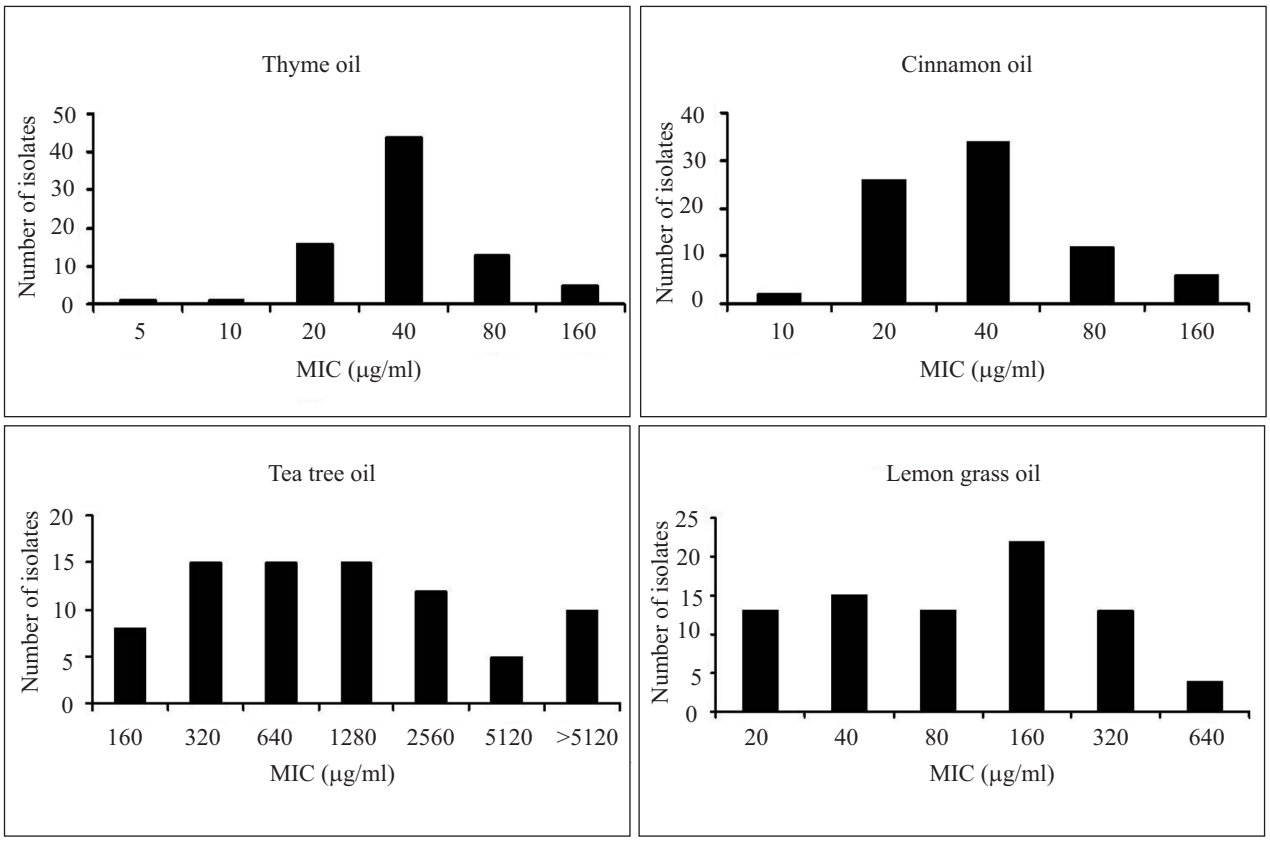

Fig. 1. Frequencies of essential oils MICs (µg/ml) for *N. seriolae* isolates (n = 80) determined by microplate resazurin assay.

The checkerboard test

Among the activities of the four studied EOs, thyme and cinnamon EOs demonstrated the lowest MICs against *N. seriolae* isolates and hence were used for the checkerboard test. The activity panel of cinnamon-thyme EOs combination against *N. seriolae ATCC43993* (Fig. 2) showed thyme EO alone with MIC 20 µg/ml, cinnamon EO alone with MIC 40 µg/ml and cinnamon-thyme EO combination with MIC 10 µg/ml for both EOs. This combination demonstrated a synergistic effect with an FIC index of 0.75. For *N. salmonicida ATCC27463,* The combination activity panel (Fig. 3) showed thyme EO alone with MIC 160 µg/ml, cinnamon EO alone with MIC 160 µg/ml and cinnamon-thyme EO combination with MIC 80 µg/ml. This combination demonstrated an additive effect with an FIC index of 1.0. For *N. asteroides ATCC19247*, the combination activity panel (Fig. 4) showed thyme EO alone with MIC 20 µg/ml, cinnamon EO alone with MIC 160 µg/ml and cinnamon-thyme EO combination with MIC 20 µg/ml for both tested EOs. This combination demonstrated an indifference effect with an FIC index of 1.125.

Discussion

The antibiotic resistance effect on the environment has led to the restoration of EOs significance. This study emphasizes the antimicrobial activities of cinnamon, thyme, lemongrass and tea tree EOs alone and in combination against clinical and standard fish Nocardiosis isolates. *In vitro* results of this work showed that all tested EOs had antibacterial activity against *N. seriolae* isolates. Among the tested EOs, cinnamon and thyme oils were both found to exhibit the lowest MIC values (5-160 µg/ml). Our result of strong activity of cinnamon EO against *N. seriolae* isolates was in accordance with a previous study (Bady and Mahdi 2014) against *Nocardia* isolates from human respiratory infections in which cinnamon oil was one of the most promising EOs with an MIC of 1.25(v/v). Other studies, (Smith-Palmer et al. 1998, Rusenova and Parvanov 2009) revealed that cinnamon and thyme oils were active against Gram-positive and Gram-negative bacteria and fungi of veterinary importance like *S. enterica, E. coli, S. aureus* and *L. monocytogenes.* The antimicrobial activity of the EO of cinnamon has been related to its cinnamaldehyde content, while the antimicrobial activity of *Thymus* species has been attributed to their phenolic compo-

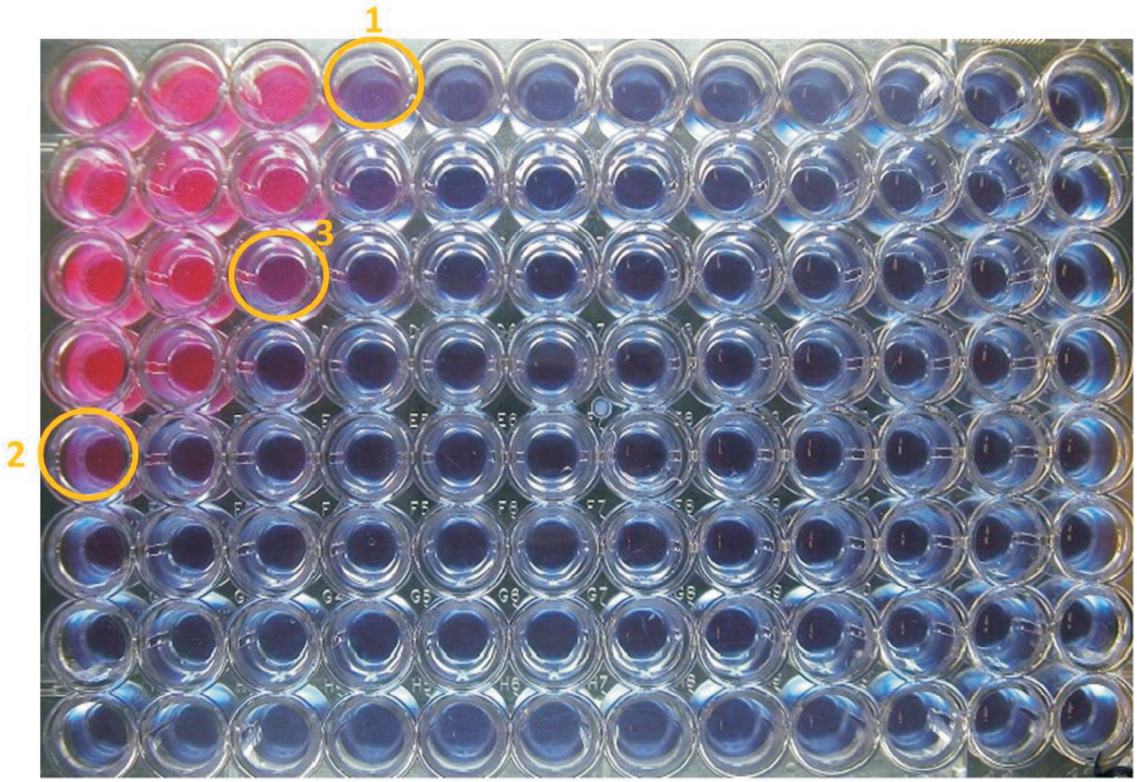

Fig. 2. The cinnamon-thyme EOs checkerboard panel against *N. seriolae ATCC43993* showing thyme EO alone with MIC 20 µg/ml (well 1), cinnamon EO alone with MIC 40 µg/ml (well 2), and cinnamon-thyme EO combination with MIC 10 µg/ml for both (well 3).

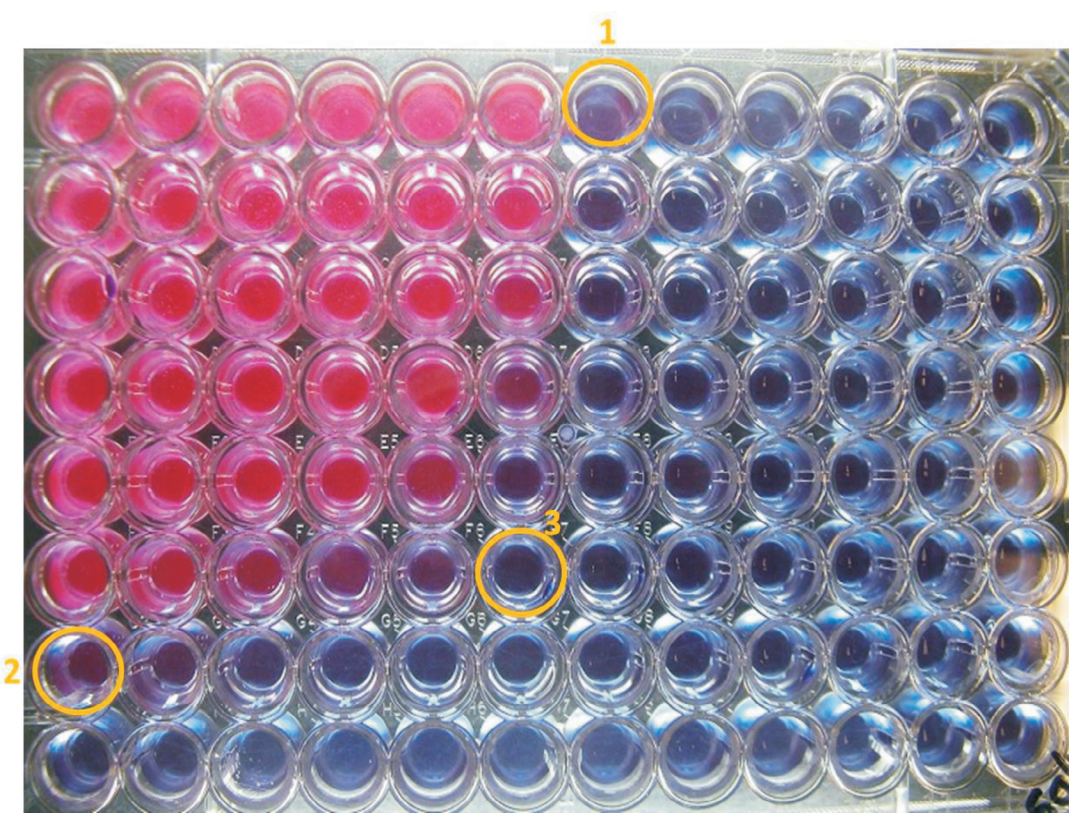

Fig. 3. The cinnamon-thyme EOs checkerboard panel against *N. salmonicida ATCC27463* showing thyme EO alone with MIC 160 µg/ml (well 1), cinnamon EO alone with MIC 160 µg/ml (well 2), and cinnamon-thyme EO combination with MIC 80 µg/ml for both (well 3).

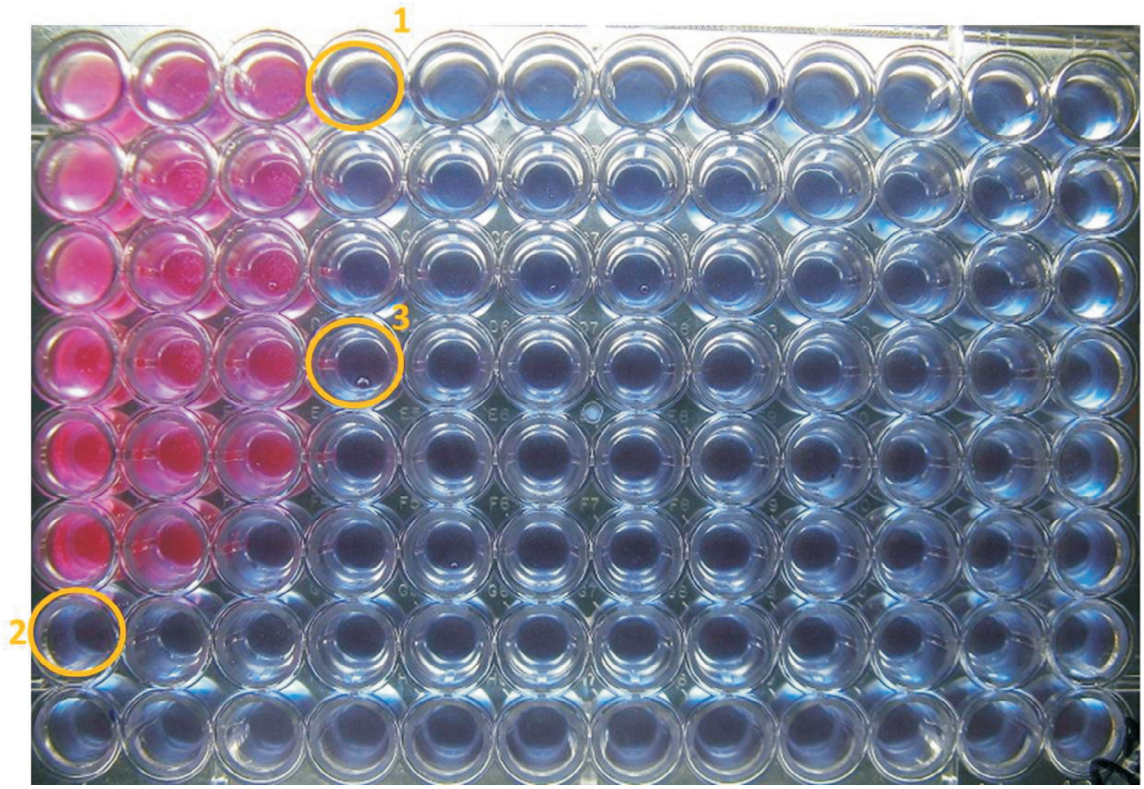

Fig. 4. The cinnamon-thyme EOs checkerboard panel against *N. asteroides ATCC19247* showing thyme EO alone with MIC 20 µg/ml (well 1), cinnamon EO alone with MIC 160 µg/ml (well 2), and cinnamon-thyme EO combination with MICs 20 µg/ml for both (well 3).

nents such as thymol and carvacrol (Prabuseenivasan et al. 2006). It has been reported that EOs containing aldehydes or phenols, such as cinnamaldehyde, carvacrol, eugenol or thymol as major components showed the highest antibacterial activity (Bassole and Juliani 2012). For lemongrass and tea tree EOs, our study showed moderate activity of lemongrass with MIC 20-640 µg/ml followed by the low activity of tea tree oil against the tested *N. seriolae* isolates with MIC of 160->5120 µg/ml. These results are in agreement with Rattanachaikunsopon and Phumkhachorn (2010) who recorded that cinnamon oil and lemongrass oil have the strongest and weakest antimicrobial activities against *S. iniae* infection in tilapia with MIC values of 40 and 320 µg/ml, respectively. The results of EOs studies could differ because of many uncontrollable variables including the plant chemotype, plant harvest and storage conditions and not necessarily in terms of actual antibacterial activity (Hammer et al. 1999).

Combinations of EOs provide an effective and economically feasible approach in combating antibiotic resistant bacteria. However, unlike studies on antibiotic-antibiotic combinations, combinations of EOs are not so widely investigated. The EO of *T. vulgaris* demonstrated good antimicrobial properties; however, the activity of thyme oil in combinations with other EOs is not well investigated. In the present study, we investigated the activity of a combination of thyme oil with cinnamon oil against the representatives of fish Nocardial spp – *N. seriolae ATCC43993*, *N. salmonicida ATCC27463*, and *N. asteroides ATCC19247*. Against *N. seriolae ATCC43993,* the present study has demonstrated a synergistic effect between thyme and cinnamon EOs with FIC index of 0.75. These results were similar to the patterns found against *S. aureus* in which the synergistic effect was found in a combination of *T. vulgaris* and *Cinnamomum zeylonicum* EOs (Kon and Rai 2012). Synergism between thyme and cinnamon EOs against *N. seriolae ATCC43993* may be caused either by not well understood interactions between cinnamaldehyde and thyme EO components, or by already documented synergistic interactions against another gram-positive bacterium, *L. monocytogenes,* between carvacrol of thyme oil and eugenol of cinnamon oil, between thymol of thyme oil and eugenol, and between thymol and linalool of cinnamon oil (Bassole et al. 2010). Against *N. salmonicida ATCC27463,* the present study has demonstrated an additive effect with an FIC index of 1.0. These results are in accordance with a previous study on other microorganisms in which

the combination of cinnamon oil and thyme oil exhibited an additive effect against *B. subtilis*, *B. cereus* and *S. aureus, E. coli* and *S. typhimurium* (Fei et al. 2011). Against N. *asteroides ATCC19247,* the present study has demonstrated an indifference effect with an FIC index of 1.125.

The combination of a pair of EOs showing synergistic or additive effects will increase the activity of both agents or reduce the concentration needed to yield the same antimicrobial effect when compared with the sum of the purified components. The practical implications of these observations are important at the time of using EOs as food additives, since the use of the lower concentration needed to yield a similar antibacterial activity will mean reduced flavor notes in fish food. Another benefit, which requires further study, of the combination of thymol, carvacol and cinnamaldehyde is that a considerable reduction in MIC's of the antibiotics were recorded when paired combinations of them and antibiotics were used.

The result of these *in vitro* tests indicates that the combination of thyme oil and cinnamon oil alone or at a given ratio has a promising potent clinical significance in the treatment of fish nocardiosis. Despite the promising results given by in vitro studies, the clinical benefits of these EOs combinations can only be determined through carefully designed *in vivo* experimental studies.

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