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Antibacterial Activity of Thyme, Laurel, Rosemary and Parsley Essential Oils Against Some Bacterial Fish Pathogen

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Research Article

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Abstract

In this study, the antibacterial activities of the essential oils of thyme (*Thymus vulgaris* L.) (TEO), laurel (*Laurus nobilis* L.) (LEO), rosemary (*Rosmarinus officinalis* L.) (REO) and parsley (*Petroselinum crispum* L.) (PEO) against different fish pathogens such as *Yersinia ruckeri* two strains (a & b), *Lactococcus garvieae, Pseudomonas fluorescens, Aeromonas sobria, Aeromonas salmonicida* and *Aeromonas veronii* were investigated. The essential oils had been extracted by hydro-distillation using a Clevenger apparatus, and their antibacterial activities were measured by paper disk diffusion method. The antibacterial activities of essential oils showed significant differences depending on the plant source and on bacterial strain. All essential oils showed inhibitory effect against bacterial fish pathogens (except PEO against *Y. ruckeri* (b)), and the inhibition zones ranged from 6.00 to 36.00 mm. The highest antibacterial activity against all tested bacteria was determined in TEO with a diameter of inhibition zone ranging from 26.50 to 36.0 mm, while LEO and REO showed a moderate activity with a diameter of inhibition zone ranging from 9.50 to 18.50 mm. The PEO illustrated the lowest diameter of inhibition zone against all the test bacteria. Among the tested bacteria, *A. veronii* was the most sensitive to the inhibitory activity of TEO, LEO and REO, while *P. fluorescens* was the most resistant. The results suggested that essential oils from thyme, laurel and rosemary can be used as natural antibacterial agents against bacterial fish pathogens.

Key words*:* Köyceğiz Lagoon, Turkish wooden dalyan, grey mullet, growth

Kekik, Defne, Biberiye ve Maydanoz Uçucu Yağlarının Bazı Bakteriyel Balık Patojenlerine Karşı Antibakteriyel Aktivitesi

Özet

Bu çalışmada, kekik (*Thymus vulgaris* L.) (KUY), defne (*Laurus nobilis* L.) (DUY), biberiye (*Rosmarinus officinalis* L.) (BUY) ve maydanoz (*Petroselinum crispum* L.) uçucu yağlarının (MUY) *Yersinia ruckeri* (a ve b), *Lactococcus garvieae, Pseudomonas fluorescens, Aeromonas sobria, Aeromonas salmonicida* and *Aeromonas veronii* gibi farklı balık patojenlerine karşı antibakteriyel aktiviteleri incelenmiştir. Uçucu yağlar Clevenger cihazı kullanılarak hidro-destilasyon yöntemi ile elde edilmiş ve antibakteriyel aktiviteleri kâğıt disk difüzyon metodu ile belirlenmiştir. Uçucu yağların antibakteriyel aktivitesi, bitki kaynağı ve bakteri türüne bağlı olarak önemli farklılıklar göstermiştir. Tüm uçucu yağlar balık patojen bakterilerine (*Y. ruckeri* (b)'ye karşı MUY hariç) karşı inhibitör etki göstermiş ve inhibisyon zonları 6,00 ile 36,00 mm arasında değişmiştir. Test edilen tüm bakterilere karşı en yüksek antibakteriyel aktivite, 26.50 ile 36,0 mm arasında değişen bir inhibisyon zon çapı ile KUY'da belirlenirken, DUY ve BUY, 9,50 ile 18,50 mm arasında değişen inhibisyon zon çapı ile orta düzeyde bir aktivite göstermiştir. MUY, tüm test bakterilerine karşı en düşük inhibisyon zon çapı sergilemiştir. Test edilen bakteriler arasında, *A. veronii*, KUY, DUY ve BUY'un inhibitör aktivitesine en duyarlı bakteri olurken, *P. fluorescens*, en dirençli bakteri olmuştur. Bu sonuçlar kekik, defne ve biberiyeden elde edilen uçucu yağların bakteriyel balık patojenlerine karşı doğal antibakteriyel ajan olarak kullanılabileceğini ortaya koymuştur.

Anahtar Kelimeler: Antibakterial aktivite, uçucu yağ, bakteriyel balık patojenleri

INTRODUCTION

Fish are exposed to numerous pathogen microorganisms due to contact with the aquatic environment (Stratev et al., 2018). Fish pathogens are responsible for serious diseases with heavy mortality and high economic losses in wild and cultured fish. Antibiotics are widely used for the control of pathogen microorganisms (Serrano, 2005), but their use can cause serious environmental problems due to their rapid spread in water (Soltani et al., 2009). Various problems, such as solubility, taste, toxicity and cost, limit antibiotics that can be used especially in food culture (Choudhury et al., 2005). Furthermore, bacterial resistance against antibiotics increases over time and consequently decreases the effectiveness of antibiotics (Choudhury et al., 2005; Dubber and Harder, 2008; Stratev et al., 2018). There is, therefore, a growing interest in the identification of effective, new antibacterial agents that would serve as a new agent to use preventing bacterial diseases in aquaculture (Bansemir et al., 2004; Dubber and Harder, 2008; Bulfon et al., 2014).

Essential oils have become increasingly more popular as an alternative to synthetic preservative agents (Wong and Kitts, 2006). They are produced from different parts of plants such as flower, leave, bud, root, seed, stem, fruit (Ghabraie et al., 2016) as plant secondary metabolites (Zaouali et al., 2010). Essential oils are obtained from plant parts by distillation, extraction, fermentation or expression, but the most common method is distillation (Burt, 2004). Essential oils can inhibit or slow the growth of bacteria, yeast and molds (Nazzaro et al., 2013) and some of them have been used for their anti-inflammatory, antiviral, antitumor, anti-hyperglycemic and anti-carcinogenic activities (Wei and Shibamoto, 2007). The Efficiency of essential oils is related to presence of their bioactive components (Toroğlu and Çenet, 2006). Harvesting season, type and part of plant, geographical structure and climate of the region where it is cultivated and extraction method are effect on the quantity and composition of essential oils (Burt, 2004). There are various studies on the use of antimicrobial agents derived from natural materials in aquaculture (Park et al., 2016; Metin et al., 2017). Components of essential oils affect the membrane and cytoplasm of bacterial cell, and alter their morphology, increase of permeability and leakage of vital intracellular constituents (Nazzaro et al., 2013). This effect leads to lysis and death when exceeds a limit (Vergis et al., 2015). Generally, antibacterial effect of essential oils against Gram positive bacteria is greater than Gram negative bacteria (Omonijo et al., 2018), because outer membrane coating the cell wall restrict the entrance of lipophilic compounds through the lipopolysaccharide structures (Vergis et al., 2015).

Thyme (*Thymus vulgaris* L.), laurel (*Laurus nobilis* L.), rosemary (*Rosmarinus officinalis* L.) and parsley (*Petroselinum crispum* L.) naturally grow in Turkey and are commonly used in traditional medicines (Önenç and Açıkgöz, 2005; Altunbaş and Türel, 2009). Thyme, belonging to the Labiatae family (Önenç and Açıkgöz, 2005), is known as carminative, antiseptic, antioxidant, antimicrobial and antifungal (Bozin et al., 2006; Gibriel et al., 2013) and is used in food, cosmetic and pharmaceutical industries (Nabavi et al., 2015). Laurel, a plant of the Lauraceae family, is an evergreen tree or shrub (Yilmaz et al., 2013) native to Mediterranean region (Ramos et al., 2012). Quantitatively, major components of thyme essential oil (TEO) are thymol and carvacrol (Teixeira et al., 2013), while laurel essential oil (LEO) contains 1,8-cineole, α-terpinene, sabinene, α-pinene, β-pinene (Ghabraie et al., 2016). TEO also contains p-cymene, β-myrcene, γ-terpinene and linalool (Jouki et al., 2014). It is reported that LEO has antimicrobial activity against food spoilage and pathogenic bacteria isolated from fish and shellfish (Ramos et al., 2012; Snuossi et al., 2016). Rosemary, a herb of the Lamiaceae family (Perez et al., 2007), is used as fresh, dried or essential oil (Özcan and Chalchat, 2008), and is also known as natural antimicrobial agent (Genena et al., 2008; Zaouali et al., 2010). Rosemary contains flavones, steroids, diterpenes and triterpenes, and its main compounds, responsible for antimicrobial activity, are 1,8-cineole, α-pinene, bornyl acetate and camphor (Genena et al., 2008). Parsley (*Petroselinum crispum* L.), belonging to the Apiaceae family, is a medicinal and food plant (Kurowska and Gałązka, 2006). Fresh and dried leaves of parsley are used as flavoring agent, condiment, garnish and food additives (Zhang et al., 2006; Altunbaş and Türel, 2009). Parsley essential oil (PEO) can be extracted from seed and leaves (Zhang et al., 2006), and it has been investigated as antimicrobial agents in previous studies (Wong and Kitts, 2006; Teixeira et al., 2013).

There is some information about antibacterial activity of essential oils obtained from different plants against some fish pathogens in the literature. In this study, we aimed at determining the inhibitory effect on *Yersinia ruckeri* (a and b), *Lactococcus garvieae, Pseudomonas fluorescens,* *Aeromonas sobria*, *A. salmonicida* and *A. veronii* of essential oils obtained from thyme, laurel, rosemary leaves and parsley seed.

MATERIALS and METHODS

Plant materials

Dried thyme (*Thymus vulgaris* L.), laurel (*Laurus nobilis* L.) and rosemary (*Rosmarinus officinalis* L.) leaves, and parsley (*Petroselinum crispum* L.) seeds were purchased from a local market (Samsun, Turkey), powdered by using a grinder (Sinbo, 2909 model, Istanbul, Turkey) and kept in bottles under cool condition until use.

Bacterial strains

In total 7 bacteria, *Yersinia ruckeri* strains (a & b), *Lactococcus garvieae*, *Pseudomonas fluorescens, Aeromonas sobria, A. salmonicida* and *A. veronii* strains were used as test organism. These were isolated from sick fish caught in The Black Sea Region of Turkey (Table 1) and identified with conventional culture methods and VITEK 2 automated system in Fish Diseases Laboratory (Veterinary Control Institute, Samsun, Turkey).

Table 1. The bacterial strains and origins used in the study

Bacteria	Origin
Yersinia ruckeri (a)	Rainbow trout (*Oncorhynchus mykiss*), Black Sea Region, Yakakent, Samsun (saltwater fish)
Yersinia ruckeri (b)	Rainbow trout (*O. mykiss*), Black Sea Region, Gürgentepe, Ordu (freshwater fish)
Lactococcus garvieae	Rainbow trout (*O. mykiss*), Black Sea Region, Gürgentepe, Ordu (freshwater fish)
Pseudomonas fluorescens	Rainbow trout (*O. mykiss*), Black Sea Region, Bafra, Samsun (freshwater fish)
Aeromonas sobria	Common carp (*Cyprinus carpio*), Black Sea Region, Tokat (freshwater fish)
Aeromonas salmonicida	Rainbow trout (*O. mykiss*), Black Sea Region, Bafra, Samsun (freshwater fish)
Aeromonas veronii	Common carp (*C. carpio*), Black Sea Region, Kavak, Samsun (freshwater fish)

Essential oil extraction

The essential oils used in the present study were extracted by hydro-distillation using a Clevenger apparatus (Sesim Kimya Laboratuvar, Ankara, Turkey). For this purpose, 50 g sample of each plant powder was mixed with 500 mL distilled water and distilled for 3 h. The extracted essential oils were dehydrated with anhydrous sodium sulphate, and then stored in the dark glass bottles at +4 ºC until used.

Determination of antibacterial activity

The antibacterial activity of essential oils was measured by paper disc diffusion method according to Zaouali et al. (2010). The sterile filter paper discs (6 mm diameter) were individually impregnated with 15 µL of each essential oil, placed onto the Mueller Hinton agar plates containing test bacteria organisms and incubated at 18 °C for 48 h. Four different antibiotics (gentamicin, 30 µg for *Y. ruckeri* (a) and (b), cefoperazone, 75 µg for *L. garvieae, A. salmonicida* and *A. veronii*, kanamycin, 30 µg for *P. fluorescens* and florfenicol, 30 µg for *A. sobria*) were used as a positive control and distilled water was used as negative control. After incubation, a diameter of inhibition zones around the disk was measured as mm, including the disk. All tests were performed in duplicate and results are expressed as average values of zone diameter.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) and the mean comparisons were determined by Duncan’s multiple range test. The data were expressed as mean ± standard deviation. Analysis was performed using the SPSS statistical package program (SPSS 17.0 for windows, SPSS Inc., Chicago, IL, USA). A significance level of 0.05 was chosen.

RESULTS and DISCUSSION

The antibacterial activity of thyme (TEO), laurel (LEO), rosemary (REO) and parsley essential oils (PEO) is presented in Table 2. The antibacterial activity of essential oils showed significant differences depending on plant source and on bacterial strain. There were no inhibition zones in the negative controls (distilled water), while the positive controls showed strong antibacterial activity against test microorganisms. All essential oils showed an inhibitory effect against fish pathogenic bacteria (except PEO against *Y. ruckeri* (b)), and the inhibition zones ranged from 6.00 to 36.00 mm. The highest antibacterial activity against all test bacteria was determined in TEO, and LEO and REO showed a moderate activity against all tested bacteria with a diameter of inhibition zone ranging from 9.50 to 18.50 mm. The PEO illustrated the lowest diameter of inhibition zone against all tested bacteria. The differences in diameter of inhibition zone could be attributed to the chemical components of these essential oils which could be able to disrupt and penetrate the lipid structure of the bacteria cell membrane, leading to its destruction (Peng and Li, 2014). Novak et al. (2012) and Miladi et al. (2013) reported that the antimicrobial activity efficiency of essential oil components is in the following order: phenols > aldehydes > ketones > alcohols > ethers > hydrocarbons. The main component of TEO is thymol, a monoterpene with phenolic ring (Novak et al., 2012; Miladi et al., 2013), while the main component of LEO and REO is 1,8-cineole belonging to ethers group (Celikel and Kavas, 2008; Ekren et al., 2013). However, the main component of PEO is α-pinene belonging to terpene class (Altunbaş and Türel, 2009). According to this, results of our study are in accordance with these reports, and similar results were also reported by various researchers. For example, Birinci Yıldırım and Türker (2018) reported that essential oil of parsley (*Petroselinum sativum*) leaves had weaker antibacterial activity against *A. solmanicida*, *Y. ruckeri* and *L. garvieae* than that of thyme, rosemary and laurel essential oils. In addition, thyme essential oil exhibited stronger antimicrobial effect against studied bacteria than the other essential oils. The antimicrobial activity of twelve natural extracts against two fish spoilage bacteria (*Pseudomonas fluorescens*, *Aeromonas hydrophila/caviae*) was tested by Iturriaga et al. (2012) with paper disc diffusion method, and diameters of inhibition zone of REO against *P. fluorescens* and *A. hydrophila/caviae* were determined as 1.0 and 16.0 mm, while diameters of inhibition zone of TEO against same microorganisms were determined as 5.3 and 33.0 mm, respectively. However, PEO showed 3.3 mm diameter of inhibition zone against *A. hydrophila/caviae* and showed no inhibition zone against *P. fluorescens*. Some plant ethanolic extracts were screened for antibacterial activity against some fish pathogens (*Listonella anguillarum*, *Photobacterium damselae* subsp. *piscicida*, *Yersinia ruckeri*, *Lactococcus garvieae*), and *Rosmarinus officinalis* indicated weak antimicrobial activity with inhibition zones 0.3 and 4.3 mm against *Y. ruckeri and L. garvieae*, respectively. But, *Thymus vulgaris* showed no inhibitory activity against *L. garvieae*, and demonstrated inhibition zone with 0.5 mm on growth of *Y. ruckeri* (Bulfon et al., 2014). In the study used the disk diffusion method, Starliper et al. (2015) reported that the diameters of inhibition zone of 20% emulsions of TEO and REO against *Aeromonas salmonicida* subsp. *salmonicida* was 42.0 and 10.7 mm, respectively. *L. nobilis* (LEO), *A. graveolens* and *Z. officinale* essential oils were tested against several bacterial strains (*A. hydrophila*, *E. cloacae*, *K. ornithinolytica*, *K. oxytoca*, *S. lentus*, *S. lugdunensis*, *S. odorifera*, *S. sciuri*, *S. xylosus*, *V. alginolyticus*) isolated from fish and shellfish, and *L. nobilis* essential oil possesses the highest antibacterial activity (mean= 14.25 mm, including disc diameter) against all tested bacteria (Snuossi et al., 2016). Tural and Turhan (2017) investigated the antimicrobial activity of TEO, REO, LEO and their mixtures, and reported that the highest activity against *S. aureus, E. coli* O157:H7 and *L. monocytogenes* was determined in TEO with zone diameters of 39.33, 28.00 and 30.67 mm, respectively. Similar results were also reported by Saricaoglu and Turhan (2018).

Table 2. Antibacterial activity of thyme (TEO), laurel (LEO), rosemary (REO) and parsley essential oils (PEO) against some fish pathogens by paper disc diffusion method

Essential oils	**Diameter of inhibition zone (mm)**						
	Y. ruckeri (a)	*Y. ruckeri* (b)	*L. garvieae*	*P. fluorescens*	*A. sobria*	*A. salmonicida*	*A. veronii*
TEO	31.50±0.5^{a,B}	29.50±0.5^{a,C}	29.50±0.5^{b,C}	26.50±0.5^{b,D}	31.50±0.5^{b,B}	30.00±1.0^{a,BC}	36.00±2.0^{b,A}
LEO	11.50±0.5^{c,D}	11.50±0.5^{c,D}	18.50±0.5^{c,A}	9.50±0.5^{c,E}	15.00±1.0^{d,B}	13.00±1.0^{d,C}	18.50±0.5^{c,A}
REO	10.00±1.0^{d,D}	10.50±0.5^{d,D}	13.00±1.0^{d,C}	10.00±1.0^{c,D}	17.00±1.0^{c,A}	14.50±0.5^{c,B}	17.50±0.5^{c,A}
PEO	7.00±0.0^{e,AB}	0±0.0^{e,D}	6.00±0.0^{e,C}	6.50±0.5^{d,B}	7.00±0.0^{e,AB}	7.50±0.5^{e,A}	7.00±0.0^{d,AB}
Negative control[1]	0±0.0^{f}	0±0.0^{e}	0±0.0^{f}	0±0.0^{f}	0±0.0^{f}	0±0.0^{f}	0±0.0^{e}
Positive control	14±1.0^{b,2}	18.0±0.0^{b,2}	32.5±0.5^{a,3}	28.5±0.5^{a,4}	37.5±1.5^{a,5}	21.5±0.5^{b,3}	40.5±1.5^{a,3}

All values are mean ± SD of duplicate.
Means with different superscripts in column wise (lower case alphabet) and a row wise (upper case alphabet) differ significantly ($p < 0.05$).
[1]Distilled water
[2]Gentamicin (30 µg)
[3]Cefoperazone (75 µg)
[4]Kanamycin (30 µg)
[5]Florfenicol (30 µg)

As seen in Table 2, among the tested bacteria, *A. veronii* was the most sensitive to the inhibitory activity of TEO, LEO and REO, while *P. fluorescens* was the most resistant. Similar to *P. fluorescens*, the growth of *Y. ruckeri* (a) and (b) was weakly inhibited by REO. However, the growth of *L. garvieae* and *A. sobria* was strongly inhibited by LEO and REO, respectively. *Y. ruckeri* (a), *L. garvieae*, *P. fluorescens*, *A. sobria, A. salmonicida* and *A. veronii* showed weak sensitivity to PEO, but *Y. ruckeri* (b) did not show any sensitivity. The results of our study are consistent with the available literature reporting that the antibacterial activity of essential oils depends on bacterial strain. Similar to our results, *L. garvieae* was found to more sensitive to the inhibitory activity of laurel essential oil than *Y. ruckeri* and *A. solmonicida* (Birinci Yıldırım and Türker, 2018). The antimicrobial activity of forty-six native Brazilian plant methanol extracts against fish pathogenic bacteria (*Streptococcus agalactiae, Flavobacterium columnare, Aeromonas hydrophila*) using the agar diffusion method was evaluated by Castro et al. (2008), and thirty-one extracts of them showed antimicrobial activity for at least one strain tested in the study. *Flavobacterium columnare* was determined as the most susceptible microorganism to major of the extracts used the study. Iturriaga et al. (2012) tested the antimicrobial activity of various naturel extracts against *P. fluorescens* and *A. hydrophila/caviae*) and reported that *P. fluorescens* was the most resistant strain. Bulfon et al. (2014), some plant ethanolic extracts were screened for antibacterial activity against some fish pathogens (*Listonella anguillarum*, *Photobacterium damselae* subsp. *piscicida*, *Yersinia ruckeri*, *Lactococcus garvieae*), and *Y. ruckeri* was determined as the most resistant bacteria for these extracts. Essential oils of clove, peppermint and lavender were tested for the inhibition effect against *L. garvieae*, *Y. ruckeri* and *A. sobria*, and *A. sobria* was the most sensitive bacteria to the inhibitory activity of all the test essential oils (Metin et al., 2017). Previous studies showed that gram-negative bacteria are more resistant to hydrophobic antibacterial agents than gram-positive bacteria (Wong and Kitts, 2006; Omonijo et al., 2018). This mainly attributed to entrapment of hydrophobic agents at the outer lipopolysaccharide layer, or delaying the adverse effect of hydrophobic agents on the cell membrane (Wong and Kitts, 2006). The results obtained from our study showed that among the essential oils, the highest antimicrobial activity was determined in thyme essential oil, and it inhibited the growth of both Gram negative and Gram positive bacteria.

CONCLUSION

The present study showed that TEO exhibited the highest antibacterial activity against the bacterial fish pathogen, and LEO and REO exhibited a moderate antibacterial activity, whereas PEO illustrated the lowest diameter of inhibition zone against all the test bacteria. The results suggested that essential oils from thyme, laurel and rosemary can be a natural and cheaper alternative treatment for controlling bacterial diseases in aquaculture.

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