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# Effect of Rosemary (*Rosmarinus officinalis*) and Sage (*Salvia officinalis*) Essential Oils on Disease Resistance against *Aeromonas sobria* in Goldfish (*Carassius auratus)*

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**Keywords**: goldfish, rosemary, sage, essential oil, disease resistance, *Aeromonas sobria*

**Abstract**

In this study, the chemical composition and the antibacterial properties against *Aeromonas sobria* of the essential oils of rosemary and sage in gold fish were evaluated *in vitro* and *in vivo* for the first time. The major constituents were eucalyptol (26.36 %) and camphor (29.17 %) for rosemary essential oil and eucalyptol (27.08 %), thujone (20.52 %) and camphor (16.99 %) for sage oil. In vitro antibacterial effects of essential oils against *A. sobria* were detected by agar diffusion assay. The in vitro antibacterial effect of rosemary (20±2.82 mm) and sage (18.5±2.12 mm) essential oils (1000 µl $ml^{-1}$) were determined to be strong against *A. sobria* ($p<0.05$). The effects of the essential oils on the disease resistance of goldfish were also investigated. Fish (5±0.15 g) were fed with different concentrations (0, 1.0 and 3.0 ml $kg^{-1}$) of each essential oil for 45 days. After feeding, the fish were challenged with *A. sobria*. Dietary administration of sage essential oil (1.0 and 3.0 ml $kg^{-1}$) and rosemary oil (3 ml $kg^{-1}$ ) resulted in a significant reduction in mortality due to *A. sobria* compared to control fish. Our results suggest that these plant oils could provide an alternative for *A. sobria* infection control in aquaculture.

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## Introduction

Aquarium fish farming has economic importance and value in the aquaculture sector in recent years. The spread of infectious diseases has accelerated due to the increase in imports and exports of aquarium fish worldwide. So, fish diseases are among the factors limiting aquarium fish farming (Lievens et al., 2011; Mankhakhet et al., 2012). Bacterial infections are often seen in aquarium fish causing significant economic losses. When preventive measures are not taken, the disease is easily transmitted to other fish.

Motile aeromonad septicaemia (MAS), is probably the most common bacterial disease in freshwater aquarium fish (Lewbart, 2001; Jagoda *et al.,* 2014). Among the motile aeromonads, *Aeromonas hydrophila*, *A. sobria* and *A. caviae* are most commonly associated with fish. *A. hydrophila* has gained much attention as the most common fish pathogenic motile Aeromonas species, but other Aeromonas species may also play an essential role in infection (Beaz-Hidalgo and Figueras, 2012). The goldfish, *Carrassius auratus* has high susceptibility to Motile aeromonads (Citarasu et al., 2011). It is often an opportunistic infection, characterized by non-specific signs such as fin rot, ulceration, haemorrhages, exophthalmia and dropsy (Beaz-Hidalgo and Figueras, 2012).

Antibiotics are commonly used for the treatment of fish infections in aquaculture. However, they cause significant problems including resistance development in bacteria, residue in fish tissue, suppression of immune system and damage of beneficial microbial flora (Defoirdt et al., 2007; Sapkota et al., 2008). For these reasons, alternative products have become more important for the control of fish diseases (Sivaram et al., 2004). The use of natural substances obtained from plants to control of fish diseases in aquaculture has increased (Goda, 2008; Aly et al., 2008; Ardo et al., 2008; Abdel-Tawwab, 2010).

The in vitro antibacterial activity of several essential oils has been evaluated against major fish bacterial pathogens (Ekici et al., 2011; Türker and Birinci Yıldırım, 2015; Öntaş et al., 2016; Görmez and Diler, 2017; Metin et al., 2017; Baba, 2018; Birinci Yıldırım and Türker, 2018). The essential oil of rosemary exhibited antibacterial activity against *Flavobacterium psycrophilum, Lactococcus garviae, Yersinia ruckeri, A. hydrophila, Aeromonas salmonicida, A. sobria, Streptococcus iniae, Edwardsiella tarda* and *E. ictaluri* (Ekici et al., 2011; Mahmoodi et al., 2012; Ostrand et al., 2012; Roomiani et al., 2013; Tural et al., 2019; Yılmaz et al., 2013). However, there is one study about the in vitro antibacterial effect of sage essential oil against fish pathogens: *A. hydrophila, A. salmonicida, Streptococcus agalactiae, Vibrio anguillarum, Y. ruckeri, Enterococcus faecalis* and *L. garviae* (Birinci Yıldırım and Türker, 2018).

Recently, many studies have focused on new natural antimicrobials such as essential oils. It was seen that essential oils could promote growth, develop fish health and increase the resistance to disease in fish because of their addition to fish feed (Zheng et al., 2009; Shehata et al., 2013). Few studies have been conducted on the *in vivo* antibacterial activity of rosemary and sage in bacterial fish diseases (Abutbul et al., 2004; Zilberg et al., 2010; Gültepe et al., 2014; Terzioglu and Diler, 2016).

Rosemary (*Rosmarinus officinalis* L., Lamiaceae) and sage (*Salvia officinalis* L., Lamiaceae) are two spices widely used in folk medicine for their antibacterial, antitumoral, antidiabetic and antioxidant activities (Lo Presti et al., 2005; Khedher et al., 2017). Rosemary and sage have been extensively studied for their antioxidative and antimicrobial activity. Especially, sage has shown strong antibacterial activities (Wu et al., 2012). The antimicrobial activity of *Salvia officinalis* was attributed to the presence of 1,8-cineole, a-thujone and camphor (Pierozan et al., 2009). The major components determined in *R. officinalis* essential oil were 1,8-cineol, camphor, ɑ-pinene, bornyl acetate, limonene, borneol, camphene, ɑ terpineol, linalool, β pinene, β caryophyllene and myrcene (Chávez-González et al., 2016). The antimicrobial activity of *R. officinalis* was related to the presence of ɑ-pinene, bornyl acetate, camphor and 1,8-cineole (Genena et al., 2008). The aim of the study was to determine the in vitro and in vivo antibacterial effects of rosemary (*Rosmarinus officinalis* L.) and sage (*Salvia officinalis* L.) essential oils against *Aeromonas sobria* causing Motile Aeromonas Septicemia in goldfish (*Carassius auratus*).

## Materials and Methods

*Plant materials*

In this study, sage (*Salvia officinalis* L.) and rosemary (*Rosmarinus officinalis* L.) were used as plant materials. The samples during the blooming stage were taken from the experimental farm of the Agriculture Faculty, Isparta Applied Sciences University. The plants were dried in the shadow and the stem parts were separated after drying. The leaves of sage and rosemary plants were used to obtain their essential oils.

*Essential oil isolation and analyses*

500 g plant samples in 1.5 L water were extracted by hydro-distilllation for 3 hours using Clevenger apparatus according to the standard procedure described in European Pharmacopoeia for determining the oil content (v/w %). The composition of essential oil was analyzed using gas chromatography-mass spectrometry (GC-MS).

CP-Wax 52 CB (50 m x 0.32 mm; film thickness = 0.25 µm) was used as the column for determining the essential oil components of the plants. GC/MS analysis was employed under the following conditions: Oven temperature program, 40 °C raised to 250 °C at a rate of 4 °C/ min and then kept at 250°C for 5 minutes. Injection block temperature 250 °C, detector temperature 250 °C, carrier gas and helium at flow rate of 20 ml/min. Each component was identified by comparison from the Wiley, Nist, Tutor, FFNSC library of mass Spectra. The component amount was determined by proportioning the relative blocks of the peak areas to the total peak area.

*In vitro antibacterial activity*

*Well diffusion agar assay*

Well diffusion agar assay (WDAA) was used to determine the antibacterial activity of sage and rosemary essential oils against *Aeromonas sobria.* The pathogen has previously been isolated from diseased yellow tail cichlid (*Pseudotropheus acei*) by Kubilay et al. (2019). Pathogenic bacteria were grown in Tryptose Soy Broth (TSB) for 24 h at 25 °C, and 100 µL of each culture was mixed into 100 ml of melted Tryptose Soy Agar (TSA). After solidifying and drying for 15–20 minute, wells were punched (diameter=3 mm) and 30 µL of different concentrations prepared with 96% ethanol (the test concentrations: 1000, 500, 250, 125 and 62.5 µl $ml^{-1}$) added to wells in triplicates. Controls were prepared using 96% ethanol. Plates were incubated at 25°C for 24h and observed for inhibition zones around the wells. The antibacterial activity of plant extracts was interpreted as proposed by Bansemir et al. (2006). Inhibition zones >15 mm were categorized as strong activity, from 8 to 15 mm as moderate activity, and from 1 to 8 mm as weak activity.

*Fish and experimental design*

240 healthy goldfish (average weight 5±0.15 g) were produced at the a commercial aquarium farm. The health status of fish were examined and observed immediately upon arrival in the aquariums and with 15 day intervals thereafter according to standardized laboratory procedures (Austin and Austin, 1989). Fish were randomly distributed in duplicate between twelve aquariums (50 L) with stocking density of 20 fish per aquarim. After the acclimation period (15 days), fish were fed for 45 days with basal diet (commercial aquarium fish feed: crude protein 16%, crude lipid 2.5-5 %, digestible energy 2200 kcal $kg^{-1}$) (control) and basal diet supplemented with rosemary and sage essential oils at 1.0 and 3.0 ml $kg^{-1}$ concentrations. Experimental groups were fed by hand, *ad libitum* twice daily. Water was changed daily at a rate of ~10% of the total volume. Water quality parameters were measured as follows: temperature 24 °C, dissolved oxygen 6 mg $L^{-1}$.

*In vivo antibacterial activity*

*Disease resistance*

After 45 days of feeding, a challenge test was performed on each experimental group with *A.sobria*. The challenge test was done in 2 replicates where 16 fish from each group (8 fish/replicate) were transferred to aquarium. Fish were infected with *A. sobria* by i.p. injection of bacterial suspension containing $1.1x10^9$ cfu $ml^{-1}$ ($LD_{50}$ dose). Mortality was recorded daily for 15 days. The confirmation of the infection was accomplished after reisolating the bacteria from the dead fish. *A. sobria* was isolated from the liver and kidney of diseased tilapia. The relative percent survival (RPS) was calculated according to Amend (1981).

RPS = (1 - % mortality in experiment group / % mortality in control) x 100

*Statistical analysis*

Data (zone diameters and mortality rate) were assessed by one way analysis of variance ANOVA SPSS 17.0 package program (SPSS Inc., Chicago, IL, USA). Duncan's multiple range tests were used to determine the significant variation ($p<0.05$) and the significance level was chosen as $P=0.05$.

## Results

*GC / MS analysis*

The components of sage and rosemary essential oils by GC-MS are given in **Table 1**. A total of 42 components in sage and 39 in rosemary were identified. Eucalyptol (1,8-cineole) (27.08%), thujone (20.52%), camphor (16.99%), beta pinene (4.07%) in sage; eucalyptol (26.36%), camphor (29.17%), borneol (7.34%), alpha pinene (6.72%) camphene (4.95%) in rosemary were determined.

*In vitro antibacterial activity*

In this study, sage and rosemary essential oils at concentration of 1000 µl $ml^{-1}$ against *A. sobria* showed strong inhibitory effect ($p<0.05$) (**Table 2**). The maximum inhibition zone (1000 µl $ml^{-1}$) of the essential oils of sage and rosemary againts *A. sobria* were in 18.5 mm and 20 mm, respectively.

*In vivo antibacterial activity*

*Challenge test with Aeromonas sobria*

In our study, the cumulative mortality rate in all the groups except of those who received rosemary 1.0 ml $kg^{-1}$ were lower than in the control following the challenge tests. All sage essential oil groups (1.0 and 3.0 ml $kg^{-1}$) showed no mortality after challenged with *A. sobria*. Dietary administration of 3 ml $kg^{-1}$ rosemary oil significantly reduced fish mortality ($p<0.05$) (**Table 3**).

**Table 1** Essential oil major components of sage and rosemary (%)

| Components | R. Time | Sage (Area %) | Rosemary (Area %) |
|---|---|---|---|
| Alpha-pinene | 6.70 | 3.06 | 6.72 |
| Camphene | 7.28 | 3.67 | 4.95 |
| Beta-pinene | 8.33 | 4.02 | 1.36 |
| Beta-myrcene | 8.78 | 1.61 | 0.94 |
| Cymole | 10.35 | - | 1.79 |
| Limonene | 10.60 | 0.88 | 2.03 |
| Eucalyptol (1.8-cineole) | 10.74 | 27.08 | 26.36 |
| Linalool | 14.67 | 3.84 | 2.70 |
| Thujone | 14.77 | 20.52 | 0.12 |
| Camphor | 17.02 | 16.99 | 29.17 |
| Borneol | 18.65 | 2.67 | 7.34 |
| Terpineol-alpha | 20.14 | 1.02 | 2.94 |
| Berbenone | 20.79 | - | 1.86 |
| Linalyl acetate | 23.72 | 1.13 | 0.70 |
| Bornil acetate | 25.84 | 0.75 | 2.83 |
| Caryophyllene | 34.42 | 3.32 | 1.49 |

**Table 2** Antibacterial activity of sage and rosemary essential oils against *A. sobria* (inhibition zone diameter, mm)

| Concentration (µl ml$^{-1}$) | Sage | Rosemary |
|---|---|---|
| 1000 | 18.5±2.12$^{b}$ | 20±2.82$^{c}$ |
| 500 | 10.5±2.12$^{a}$ | 16±5.65$^{abc}$ |
| 250 | 10.5±0.7$^{a}$ | 18±5.65$^{bc}$ |
| 125 | 7$^{a}$ | 8.5±0.7$^{ab}$ |
| 62.5 | 7±1.41$^{a}$ | 7.5±0.7$^{a}$ |

Different lowercase letters indicate differences between within each plant extract ($p<0.05$).

**Table-3.** Resistance of goldfish fed with different concentrations of essential oils to *A. sobria*

| Groups | Mortality (%) | RPS |
|---|---|---|
| Rosemary 1.0 ml kg$^{-1}$ | 50$^{A}$ | - |
| Rosemary 3.0 ml kg$^{-1}$ | 25$^{B}$ | 50 |
| Sage 1.0 ml kg$^{-1}$ | 12.5$^{B}$ | **75** |
| Sage 3.0 ml kg$^{-1}$ | 12.5$^{B}$ | **75** |
| Control | 50$^{A}$ | - |

Capital superscripts denote a significant difference between values for groups at cumulative per cent mortality (i.e., differences within columns).

## Discussion

The antibacterial activities of essential oils of medicinal plants have been known for a long time. These activities depend on the type, composition and concentration, processing and storage conditions of the essential oil and on the type of microorganisms and composition of the substrate (Bertini et al., 2005). In this study, forty-two compounds were identified in the essential oil of sage. The 4 major component were eucalyptol (1,8-cineole) (27.08%), thujone (20.52%), camphor (16.99%), beta-pinene (4.07%). Other authors have reported sage essential oil with a comparable composition although there are some differences regarding the quantity of the individual compounds (Kırıcı et al., 1996; Delamare et al., 2007; Said-Al Ahl et al., 2015; Craft et al., 2017; Khedler et al., 2017; Çelik et al., 2018; Karik et al., 2018). The rosemary essential oil isolated was mainly composed of five compounds: eucalyptol (26.36%), camphor (29.17%), borneol (7.34%), alpha pinene (6.72%) camphene (4.95%) in this study. A similar composition was observed for the rosemary essential oil obtained by other studies (Shiwakoti et al., 2016; Tomi et al., 2016; Hendawy et al., 2017; Yıldırım, 2018).

Rosemary and sage essential oils have been reported to be highly antimicrobial properties in numerous investigations and have also been shown to be inhibitory to pathogens (Teixeira et al., 2013). The antibacterial activity of rosemary and sage essential oils has been evaluated against major fish bacterial pathogens (Ekici et al., 2011; Mahmoodi et al., 2012; Ostrand et al., 2012; Roomiani et al., 2013; Yılmaz et al., 2013; Birinci Yıldırım and Türker, 2018). In this study, rosemary essential oil at concentration of

1000 µl $ml^{-1}$ (20 mm inhibition zone) against *A. sobria* showed strong inhibitory effect ($p<0.05$). Similarly, Tural et al. (2019) observed that essential oil of *R. officinalis* showed inhibitory effect against *A. sobria* (17 mm inhibition zone). In another study, the rosemary essential oil showed antibacterial effect against *L. garvieae* with a 24 mm inhibition diameter zone (Mahmoodi et al., 2012). Roomiani et al. (2013) also reported a strong antibacterial effect with inhibition zone of 45 mm with rosemary essential oil against *S. iniae*. In addition, Birinci Yıldırım and Türker (2018) noted that a strong inhibition effect of the rosemary essential oil againts *A. hydrophila, A. salmonicida, V. anguillarum, Y. ruckeri, L. garviae* and *S. algalactiae.* However, Ekici et al. (2011) noted that rosemary essential oil did not show antibacterial effect on *Y. ruckeri, A. hydrophila, V. anguillarum, F. psychrophilum* and *L. garvieae.*

Sage essential oil was observed inhibitory effect against *A. sobria* at a concentration of 1000 µl $ml^{-1}$ (18.5 mm inhibition zone) in this study ($p<0.05$). Similarly, Birinci Yıldırım and Türker (2018) reported the best antibacterial effect of sage oil againts *E. faecalis* and *L. garvieae*.

In the present study, rosemary oil (3.0 ml $kg^{-1}$) reduced mortality in goldfish after challenged with *A. sobria*. Similarly, several studies reported that rosemary significantly reduced mortality from *S. iniae* in tilapia (Abutbul et al., 2004; Zilberg et al., 2010; Gültepe et al., 2014).

Sage oil at 1.0 and 3.0 ml $kg^{-1}$ concentrations reduced mortality from *A. sobria* in goldfish in this study. In contrast, Terzioglu and Diler (2016) reported that dried sage powder (*S. officinalis*) did not protected against *V. anguillarum* infection in rainbow trout.

As a result of this study, in vitro antibacterial effects of sage oil and in vivo antibacterial properties of rosemary and sage essential oils in goldfish against *Aeromonas sobria* were reported for the first time. Sage essential oil at 1.0 and 3.0 ml $kg^{-1}$ concentrations and rosemary oil at 3.0 ml $kg^{-1}$ showed disease resistance against *Aeromonas sobria* infection in goldfish. These results indicated that sage and rosemary oils appears to have therapeutic potential in goldfish aquaculture. In future studies, the effects of different derivatives of these medicinal plants against different pathogens in fish species can be investigated.

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