Journal of Advanced Research (2015) 6, 89–97

Cairo University

Journal of Advanced Research

ORIGINAL ARTICLE

An investigation of the bactericidal activity of selected essential oils to *Aeromonas* spp.

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ARTICLE INFO

Article history:
Received 31 October 2013
Received in revised form 20 December 2013
Accepted 23 December 2013
Available online 3 January 2014

Keywords:
Essential oil
Aeromonas
Bactericidal
MBC

ABSTRACT

Diseases of fishes caused by *Aeromonas* spp. are common, have broad host ranges and may cause high mortality. Treatments of captive-reared populations using antimicrobials are limited with concerns for bacterial resistance development and environmental dissemination. This study was done to determine whether selected plant-derived essential oils were bactericidal to *Aeromonas* spp. Initially, twelve essential oils were evaluated using a disk diffusion assay to an isolate of *A. salmonicida* subsp. *salmonicida*, cause of fish furunculosis. The greatest zones of inhibition were obtained with oils of cinnamon *Cinnamomum cassia*, oregano *Origanum vulgare*, lemongrass *Cymbopogon citratus* and thyme *Thymus vulgaris*. Minimum bactericidal concentrations (MBC's) were determined for these four oils, Allimed® (garlic extract, *Allium sativum*) and colloidal silver to sixty-nine isolates representing nine *Aeromonas* spp. The lowest mean MBCs (0.02–0.04%) were obtained with three different sources of cinnamon oil. MBCs for three sources of oregano and lemongrass oils ranged from 0.14% to 0.30% and 0.10% to 0.65%, respectively, and for two thyme oils were 2.11% and 2.22%. The highest concentration (5%) of Allimed® tested resulted in MBCs to twelve isolates. A concentration of silver greater than 15 mg/L would be required to determine MBCs for all but one isolate.

Introduction

In fisheries and aquaculture, an effective disease treatment that would be an alternative to standard antimicrobial therapy would be beneficial in eliminating drug resistance development and environmental contamination. Diseases to fishes caused by *Aeromonas* spp. are common, have broad host ranges and may cause high mortality. For example, furunculosis, caused by *A. salmonicida* subsp. *salmonicida*, is a serious bacterial disease to

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Peer review under responsibility of Cairo University.

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http://dx.doi.org/10.1016/j.jare.2013.12.007

cultured and free-ranging salmonid fish species. Mortality may be very high and with near 100% morbidity in affected populations [1]. This disease is routinely treated with antimicrobials, but is met with varying degrees of successes. Other *Aeromonas* spp., primarily *A. hydrophila* and *A. veronii* bv. *sobria*, are also common causes of fish diseases. Three antimicrobials are approved to treat diseases in fishes caused by *Aeromonas* spp. (US Food and Drug Administration, Center for Veterinary Medicine; www.fda.gov/cvm). However, strict guidelines on the usage labels limit treatments to diseases caused by *A. salmonicida* and *A. hydrophila*, and only among certain hosts. Romet-30®, Aquaflor® and Terramycin® are approved to treat furunculosis in salmonid fishes, and Terramycin® may be used to treat bacterial hemorrhagic septicemia in catfish caused by *A. hydrophila* (formerly *A. liquefaciens*).

One of the early behavioral changes in fishes affected by most bacterial diseases is the loss of appetite [2]. Inappetence is particularly apparent in captive reared fishes where changes can be observed and is a sign common to nearly all bacterial diseases, including diseases caused by *Aeromonas*. Inappetence confounds a successful treatment with antimicrobials since the medication is delivered orally via medicated food. The time between when a disease is initially suspected or diagnosed and when fish lose appetite may be rather short and not allow for critical microbiological assessments to ensure causative bacterial isolate identification and antimicrobial susceptibility testing. Failure to ensure isolate susceptibility may contribute to the development of drug resistant bacterial isolates. *Aeromonas salmonicida* is particularly troublesome in this regard [3–5]. Furthermore, the accumulation of chemicals, such as antimicrobials, in watersheds and their exposure to wild fish populations has led to heightened concerns about their use and eventual disposition in open-water environments [3–6].

Microbiological studies have shown that certain plant-derivative essential oils are antibacterial, including *Aeromonas* spp. For instance, Hammer et al. [7] showed that oregano (*Origanum vulgare*), lemongrass (*Cymbopogon citratus*) and thyme (*Thymus vulgaris*) yielded minimum inhibitory concentrations (MICs) of 0.12% to *A. sobria*. Nya et al. [8] determined that allicin (garlic *Allium sativum*, Allimed®) had a MIC_{50} of 450 μL/mL to *A. hydrophila*. Perhaps more important, feeding rainbow trout *Oncorhynchus mykiss* with an extract of garlic in their diet for fourteen days significantly increased their survival to an experimental challenge with *A. hydrophila* [9]. The present study was done to determine whether selected plant-derived essential oils were bactericidal to various *Aeromonas* spp., including *A. salmonicida* subsp. *salmonicida*, perhaps to provide an alternative to antimicrobials for the treatment of *Aeromonas* diseases of fishes. Colloidal silver was included in this study because of a previous report of the inhibitory effect of silver to *A. hydrophila* [10].

Material and methods

Bacteria

The *A. salmonicida* subsp. *salmonicida* (isolate BD-05-08) recovered from kidney tissue from a chinook salmon *Oncorhynchus tshawytscha* from the Salmon River, NY in 2005 was used in disk diffusion testing of the essential oils. Sixty-nine *Aeromonas* isolates used for determinations of minimum bactericidal concentrations (MBCs) were collected in 2008–2011 from fish tissues from disease diagnostic cases or from the surfaces of unfertilized lake sturgeon *Acipenser fulvescens* eggs. Isolate origins are presented in Table 1. The fishes involved in the diagnostic cases from which the samples were collected were Atlantic salmon *Salmo salar*, lake trout *Salvelinus namaycush*, smallmouth bass *Micropterus dolomieu*, and green sunfish *Lepomis cyanellas*. The isolates from fish were recovered by primary streak-plate inoculations from kidney or spleen tissues or from mucus-skin or lesions. Isolates from lake sturgeon egg surfaces were recovered by placing individual eggs into sterile 0.1% peptone – 0.05% yeast extract (pH 7.0; Becton, Dickinson and Company, Sparks, MD, USA) to create a 1:10 (w/v) dilution. Each egg was vigorously mixed in the diluent using a vortex mixer (Velp Scientifica Wizard, Neu-Tec Group, Inc., Farmingdale, NY, USA) for 30 s at 75% maximum setting. The eggs remained intact during the mixing. Serial tenfold dilutions (through 1×10^{-4}) of the diluent were prepared in peptone-yeast extract broth and plate media were inoculated with 25 μL volumes from all dilutions. The bacteriological media used for primary cultures from fish were brain heart infusion agar (BHIA) and tryptic soy (TS) agar; whereas R2A medium was used to recover bacteria from the egg surfaces (BHIA, TS, R2A: Becton, Dickinson and Company, Sparks, MD, USA). All inoculated plates were incubated at 20–22 °C until bacterial colonies developed, typically 2–3 days. Single bacterial colonies were inoculated on the homologous medium to ensure purity and to develop cultures, which were archived in 20% glycerol-TS broth at −70 °C. The bacterial cultures were characterized using standard bacterial classification procedures and previously published line data for comparisons [11–14]. Motility was determined using the hanging-drop procedure [12].

Identifications of *Aeromonas* spp. to genus were accomplished with the following criteria: Gram-negative rods, fermentation of glucose, oxidase positive, reduction of nitrates, and resistance to 100 μg 2,4-diamino-6,7-diisopropylpteridine (Vibriostat; Sigma–Aldrich, Co., St. Louis, MO, USA) per mL of Mueller Hinton medium (Becton, Dickinson and Company, Sparks, MD, USA). Additional testing further characterized the isolates to species. For example, *A. veronii* bv. *sobria* was differentiated from other *Aeromonas* spp. by gas produced from glucose fermentation, positive reactions from Voges-Proskauer, arginine decarboxylase/dehydrolase, sucrose and d-mannitol, and negative test results from ornithine decarboxylase, l-arabinose, esculin hydrolysis and inositol [14].

Essential oil sensitivity – disk diffusion

A variety of plant-derived essential oils were selected for antimicrobial testing using a disk diffusion method and are listed in Table 2. The oils used included cinnamon bark (*Cinnamomum cassia*), cinnamon leaf (*Cinnamomum zeylanicum*), thyme (*T. vulgaris*), clove (*Syzygium aromaticum*), tea tree (*Melaleuca alternifolia*), rosemary (*Rosemarinus officinalis*) and basil (*Ocimum basilicum*) which were all sourced from Aromaland, Inc. (Santa Fe, NM). Lemongrass (*C. citratus*), rosewood (*Aniba rosaeodora*), sage (*Salvia officinalis*) and lavender (*Lavendula*

Table 1 Origin of sixty-nine *Aeromonas* isolates used to determine minimum bactericidal concentrations (MBC).

Aeromonas species and isolate identifier	Host and geographic origin[a]
Aeromonas salmonicida subsp. *salmonicida*: M1, M3, K4, M4, K5, K6, M6	Atlantic salmon *Salmo salar*, mucus or kidney, Vermont, 2008
Aeromonas salmonicida subsp. *salmonicida*: M7, K12, M12	Lake trout *Salvelinus namaycush*, mucus or kidney, Pennsylvania, 2011
Aeromonas hydrophila: 18b les, 19 les, 18a int	Smallmouth bass *Micropterus dolomieu*, lesions or internal tissues, Pennsylvania, 2010
Aeromonas hydrophila: SA8, SB19	Lake sturgeon *Acipenser fulvescens*, surface of unfertilized eggs, New York, 2011
Aeromonas veronii bv. *sobria*: 24 les	Green sunfish *Lepomis cyanellas*, lesion, Pennsylvania, 2010
Aeromonas veronii bv. *sobria*: 25 les, 29 int, 32 int, 47 les, 45a int, 50b les, 54 int	Smallmouth bass *Micropterus dolomieu*, lesions or internal tissues, Pennsylvania, 2010
Aeromonas veronii bv. *sobria*: SA 9	Lake sturgeon *Acipenser fulvescens*, surface of unfertilized eggs, New York, 2011
Aeromonas caviae: SG10	Lake sturgeon *Acipenser fulvescens*, surface of unfertilized eggs, New York, 2011
Aeromonas popoffii: SA2, SA5, SA15, SB3, SC1b, SC6b, SC10a, SC17, SD10, SD16, SD17, SD19, SD20, SE8a, SE9a, SE20b, SF2	Lake sturgeon *Acipenser fulvescens*, surface of unfertilized eggs, New York, 2011
Aeromonas allosaccharophila: 42 les, 49 les	Smallmouth bass *Micropterus dolomieu*, lesions, Pennsylvania, 2010
Aeromonas allosaccharophila: SB9	Lake sturgeon *Acipenser fulvescens*, surface of unfertilized eggs, New York, 2011
Aeromonas encheleia: SA4, SA14, SA16, SA17, SC20a, SE2, SE16, SE18, SE19b	Lake sturgeon *Acipenser fulvescens*, surface of unfertilized eggs, New York, 2011
Aeromonas eucrenophila: SB10, SB14, SC2b, SF4, SF5, SF10, SG1, SG2, SG4, SG5, SG16	Lake sturgeon *Acipenser fulvescens*, surface of unfertilized eggs, New York, 2011
Aeromonas molluscorum: SB13, SC9a, SC16a, SI17	Lake sturgeon *Acipenser fulvescens*, surface of unfertilized eggs, New York, 2011

[a] *Aeromonas* isolates originated from various fish (Vermont), sample collections sites from the Susquehanna River (Pennsylvania), and from various female-egg lots from the St. Lawrence River (New York).

angustifolia) were sourced from Stony Mountain Botanicals, Ltd. (Loudonville, OH, USA), and oregano (*O. vulgare*) was from North American Herb and Spice (Buffalo Grove, IL).

A sterile 1% solution of Tween-20 (polyoxyethylene sorbitan monolaurate; Sigma–Aldrich, Co., St. Louis, MO, USA) in distilled water was used to prepare 20% emulsions of each essential oil. Sterile paper disks (6 mm diameter) were saturated with approximately 25 µL of the 20% oil emulsions. Three disks per oil were placed on the surface of a freshly inoculated TS agar plates, one essential oil per plate. A fourth disk saturated with sterile 1% Tween-20 was placed on each plate to serve as a control. The inoculum for the TSA plates was prepared by growing *A. salmonicida* subsp. *salmonicida* BD-05-08 on TSA at 20 °C for 48 h. Colonial growth was suspended in 10 mL of Tween-20 to an approximate 0.5 McFarland turbidity. A sterile glass rod with a 90-degree bend was used to distribute 0.5 mL of the inoculum evenly across the surfaces of the TSA medium plates to produce a confluent lawn of growth. After the disks were applied, the plates were incubated at 20 °C for 48 h. Zones of growth inhibition were indexed as the diameter of the clear area around disks [15].

Essential oil sensitivity – minimum bactericidal concentration

The essential oils and colloidal silver tested against all *Aeromonas* isolates are presented in Table 3. Three different sources for each of oregano, cinnamon and lemongrass oils were tested to evaluate consistency of bactericidal activity from various sources. Prior to use, each essential oil and colloidal silver were filter sterilized (0.2 µm) and placed in sterile TS broth, which yielded the highest concentrations evaluated. Serial doubling dilutions were prepared, also in TS broth, from the highest concentration and 1 mL was transferred to sterile 13×100 mm tubes for inoculations with the *Aeromonas* isolates. The oil or silver dilutions series were freshly prepared and immediately inoculated with bacteria. For consistency, volumes of the essential oils and colloidal silver sufficient to evaluate all (sixty-nine) isolates simultaneously were prepared.

Table 2 Zone diameters (mm) of *Aeromonas salmonicida* subsp. *salmonicida* growth inhibition by 20% solutions of essential oils and typical percentages of major components of the essential oils.

Essential oil	20% zones, mm	Cinnamaldehyde (%)	Eugenol (%)	Other major components [reference]
Cinnamon bark *Cinnamomum cassia*	56.0 ± 0.0[aA]	61–99	13	None [20]
Oregano *Origanum vulgare*	46.0 ± 0.0[b]	nd	nd	Carvacrol, 62% [21]
Lemongrass *Cymbopogon citratus*	44.7 ± 2.3[b]	0	0.01	α and β citral, 80% [22]
Thyme *Thymus vulgaris*	42.0 ± 10.6[b]	0	0	Thymol, 40%; p-cymene, 18% [23]
Clove *Syzygium aromaticum*	29.3 ± 3.1[c]	0	87	None [30]
Cinnamon leaf *Cinnamomum zeylanicum*	27.3 ± 1.2[c]	1–2	82–85	None [31]
Rosewood *Aniba rosaeodora*	16.7 ± 4.2[d]	0	0	Linalool, 75–85% [32]
Sage *Salvia officinalis*	12.7 ± 3.1[de]	0	0–0.2	Manool, 8–21%; viridiflorol, 5–17% [33]
Lavender *Lavandula angustifolia*	12.7 ± 2.3[de]	0	0	Linalyl acetate, 43%; linalool, 33% [34]
Tea tree oil *Melaleuca alternifolia*	12.7 ± 1.2[de]	0	0	Terpinen-4-ol, 39%; γ-terpinene, 20% [34]
Rosemary *Rosmarinus officinalis*	10.7 ± 1.2[de]	Trace	Trace	*p*-Cymene, 44%; linalool, 21% [24]
Basil *Ocimum basilicum*	6.7 ± 5.8[e]	0	6	Linalool, 29%; estragole, 22% [35]
Water (control)	0.0 ± 0.0	N/A	N/A	N/A
F value, error mean square, *df*	117, 11.2, 35			

[A] Mean zone diameters ($n = 3$) not followed by a common letter (a–e) significantly differ ($P < 0.05$).

Sterile tubes containing TS broth only were inoculated with each test to test for viability of the bacterial cultures.

Each *Aeromonas* culture was prepared for susceptibility testing by thawing a frozen (−70 °C) 1 mL aliquot and transferring into 5 mL TS broth. After 24–48 h at 20 °C, 0.1 mL was transferred to 1 mL sterile TS broth and following an additional 24 h at 20 °C, 25 µL volumes were transferred to the dilution series prepared for each essential oil and colloidal silver. Viable cell enumerations were done to determine the number of bacteria (CFU/mL) in each dilution tube at the start (0 h). The tubes were incubated at 20 °C on a rotary shaker at 120 rpm. After 24 and 48 h, 10 µL was removed from each tube and placed on the surface of TSA plates.

The minimum bactericidal concentration (MBC) was determined similar to Petrus et al. [16] as the lowest essential oil or colloidal silver concentration that killed greater than 99.9% of the initial bacterial population, which was indicated by no visible bacterial growth on the TSA plate surfaces.

Statistical evaluations

Significant differences ($p < 0.05$) between mean diameters ($n = 3$) of zones of inhibition were determined using the Tukey's HSD test [17]. Computations were made using Statistix for Windows (version 8.0, 2003, Analytical Software, Tallahassee, FL 32317, USA; www.statistix.com). Significance ($p < 0.05$) of differences between mean MBCs (n = variable) were identified using Tukey–Kramer multiple comparison tests [17]. Computations were made using NCSS software [18]. Isolates in which the MBCs were greater than the highest concentration of the essential oil evaluated were not included in the data analyses.

Results

Essential oil sensitivity – disk diffusion

The mean zone diameters (mm) of inhibition of *A. salmonicida* subsp. *salmonicida* BD-05-08 by the 20% emulsions of essential oils along with percentages of major components of the oils are provided in Table 2. Zones of inhibition ranged from 6.7 ± 5.8 mm for basil oil to 56.0 ± 0.0 mm for cinnamon bark oil. The zone of inhibition for cinnamon bark oil was significantly different ($p < 0.05$) than those for all other essential oils. Zones of inhibition for oils of oregano, lemongrass and thyme did not significantly differ from each other, but were significantly larger than the zones for the remaining essential oils. Zones of inhibition for oils of clove (29.3 ± 3.1 mm) and cinnamon leaf (27.3 ± 1.2 mm) were not significantly different from each other; however, both were significantly smaller than those of oils of oregano, lemongrass and thyme (46.0 ± 0.0 mm, 44.7 ± 2.3 mm, 42.0 ± 10.6 mm, respectively). Based on these results, oils of cinnamon bark, oregano, lemongrass and thyme were selected for evaluations of MBCs to *Aeromonas* spp. The zone of inhibition by cinnamon bark oil (*C. cassia*) was significantly greater than that of cinnamon leaf oil (*C. zeylanicum*). The zone diameters for oils of rosewood (16.7 ± 4.2 mm), sage, lavender, tea tree oil (all 12.7 ± 1.2 – 3.1 mm), and rosemary (10.7 ± 1.2 mm) were not significantly different from each other, but were significantly less than the zone diameters produced by clove oil and cinnamon leaf oil.

Essential oil sensitivity – minimum bactericidal concentrations

The mean number of viable *Aeromonas* bacteria present in all essential oil-dilution tubes at time 0 h when inoculated was 2.17×10^6 CFU/tube (1 mL per tube). The sixty-nine *Aeromonas* isolates were collected from recent health and diagnostic investigations, which accounted for the variety in the number of isolates (replicates) per species, ranging from one *A. caviae* isolate to seventeen isolates of *A. popoffii*.

The highest concentration of pure essential oils tested was 5%. The highest concentration of oregano oil from Herbal Authority (13.3% purity) tested was 0.67% and that for colloidal silver solution (containing 30 mg/L silver) was 15 mg/L. All of the *Aeromonas* isolates grew in TS broth control tubes and in tubes containing the lower concentrations of essential oils, which facilitated the determinations of MBCs.

For all *Aeromonas* isolates combined, the mean percent MBCs for the essential oils are presented in Table 4. Cinnamon oils from the three different sources gave the lowest mean percent MBCs, ranging from 0.02 ± 0.02% for Lotus Brands, Inc. to 0.04 ± 0.03% for Aromaland, Inc. The MBCs for the

Table 3 Essential oils source information.

Essential oil	Highest oil concentration tested	Product concentration and source
Cinnamon, Lotus	5.0%	100% pure essential oil, *Cinnamomum cassia*. Lotus Brands, Inc., Twin Lakes, WI
Cinnamon, Frontier	5.0%	100% pure essential oil, *Cinnamomum aromaticum*. Frontier Natural Products Co-Op, Norway, IA
Cinnamon, Aromaland	5.0%	Therapeutic grade pure essential oil, *Cinnamomum cassia*. Aromaland, Inc., Santa Fe, NM
Oregano, Now Foods	5.0%	100% pure essential oil, *Origanum vulgare*. Now Foods, Bloomingdale, IL
Oregano, Stony Mountain Botanicals	5.0%	100% pure essential oil, *Origanum vulgare*. Stony Mountain Botanicals, Ltd., Loudonville, OH
Oregano, Herbal Authority	0.67%	133.33 mg/mL, *Origanum vulgare*. Herbal Authority, Holbrook, NY. Purity, 13.3%
Lemongrass, Stony Mountain Botanicals	5.0%	100% pure essential oil, *Cymbopogon citratus*. Stony Mountain Botanicals, Ltd., Loudonville, OH
Lemongrass, Now Foods	5.0%	100% pure essential oil, *Cymbopogon citratus*. Now Foods, Bloomingdale, IL
Lemongrass, Puritan's Pride	5.0%	100% pure essential oil, *Cymbopogon flexuosus*. Puritan's Pride, Inc., Oakdale, NY
Thyme white	5.0%	Therapeutic grade pure essential oil, *Thymus vulgaris* L. Aromaland, Inc., Santa Fe, NM
Thyme linalol	5.0%	Therapeutic grade pure essential oil, *Thymus vulgaris* L. ct. linalol. Aromaland, Inc., Santa Fe, NM
Allimed®	5.0%[a]	1 drop has 0.0375 mL Allisure AC-23 allicin extract of garlic *Allium sativum*. Allimax Nutraceuticals, Chicago, IL. Allicin content not defined
Colloidal silver	15 mg/L	30 mg/L, Source Naturals, Inc., Santa Cruz, CA

[a] Allimed®, 5% was based on a 0.05 mL drop volume, 3.75% AC-23.

Table 4 Overall mean minimum bactericidal concentrations (MBC) of pure essential oils to *Aeromonas* spp. Means are ranked beginning with the lowest mean percent MBC.

Essential oil	Mean MBC ± SD
Cinnamon, Lotus	0.02 ± 0.02%[a] [A]
Cinnamon, Frontier	0.03 ± 0.02%[a]
Cinnamon, Aromaland	0.04 ± 0.03%[ab]
Lemongrass, Stony Mountain Botanicals	0.10 ± 0.04%[abc]
Oregano, Now Foods	0.14 ± 0.11%[abc]
Oregano, Herbal Authority	0.16 ± 0.15%[abc]
Oregano, Stony Mountain Botanicals	0.30 ± 0.34%[bc]
Lemongrass, Now Foods	0.36 ± 0.22%[c]
Lemongrass, Puritan's Pride	0.65 ± 0.39%[d]
Thyme white	2.11 ± 1.29%[e]
Thyme linalol	2.22 ± 0.72%[e]

[A] Mean MBCs (n = 69) not followed by a common letter (a–e) significantly differ (Tukey–Kramer; $P < 0.05$).

three cinnamon oils were not significantly different from each other, nor did they differ from those of lemongrass oil (0.10 ± 0.04%) from Stony Mountain Botanicals or oregano oils (0.14 ± 0.11% to 0.16 ± 0.15%) from Now Foods and Herbal Authority. The mean MBCs for lemongrass oils from Stony Mountain Botanicals (0.10 ± 0.04%) and Now Foods (0.36 ± 0.22%) were not significantly different from the MBCs for the three oregano oils, which ranged from 0.14 ± 0.11% (Now Foods) to 0.30 ± 0.34% (Stony Mountain Botanicals). The mean MBC of lemongrass from Puritan's Pride was 0.65 ± 0.39%. The mean percent MBCs of the two thymes (white, 2.11 ± 1.29%; linalool, 2.22 ± 0.72%) did not significantly differ from each other, but both significantly differed from all essential oils.

Mean percent MBCs obtained for the individual *Aeromonas* spp. are presented in Table 5. With four of the essential oils (the three cinnamon oils and oregano oil from Now Foods), MBCs were determined for all of the isolates. With the remaining essential oils, MBCs could not be determined for all of the sixty-nine isolates at the highest concentrations of the oils tested. Discounting the results obtained using Allimed® and colloidal silver, *A. salmonicida* subsp. *salmonicida* and *A. allosaccharophila* were the only two species in which MBCs were determined for all of the isolates and all of the essential oils. With the remaining *Aeromonas* spp., MBCs could not be determined for at least one of the essential oils at the highest concentration tested. For each *Aeromonas* sp., the same isolates were responsible for the scores that required concentrations of the essential oils greater than those tested to determine MBCs. For example, with *A. popoffii*, four isolates (SE8a, SF2, SC1b, SC6b) were responsible for all of the scores in which MBCs were not determined; namely, from the three lemongrass oils, both thymes, and oregano from Herbal Authority and Stony Mountain Botanicals. All of the *Aeromonas* isolates, regardless of species, that were resistant to the greatest concentrations of the oils tested were recovered from the surfaces of lake sturgeon eggs.

The rankings of the mean percent MBCs of the various *Aeromonas* spp., which are given in Table 5, were similar to the rank when all isolates were combined, which are presented in Table 4. Generally, the three cinnamon oils gave the lowest mean percent MBCs, the two thymes required the greater concentrations with position changes in the remaining oils in between. The lowest mean percent MBC to *A. salmonicida* subsp. *salmonicida* was obtained using cinnamon oil from Lotus Brands, Inc. (0.01 ± 0.01%), which was not significantly different from eight other essential oils including lemon-

grass oil from Puritan's Pride, Inc. which had a mean of 0.31 ± 0.0%. Similarly, the same nine essential oils (cinnamon oil from Lotus Brands, Inc. through Lemongrass from Puritan's Pride) produced relatively low mean percent MBCs and were not significantly different from each other for *A. hydrophila*, *A. allosaccharophila*, and *A. molluscorum*; additionally, thyme linalool was not significantly different for *A. hydrophila*. The mean percent MBCs of the first eight essential oils listed; namely, cinnamon from Lotus Brands, Inc. through lemongrass from Now Foods, were not significantly different from each other for *A. veronii* bv. *sobria* and *A. popoffii*.

Allimed® (allicin extract of *A. sativum*) tested at 5% was ineffective at producing MBCs for most of the *Aeromonas* isolates. MBCs were recorded at 5% for seven *A. popoffii* and five *A. encheleia* isolates. Similarly, concentrations of the colloidal silver solution (containing 30 mg/L silver) greater than 15 mg/L were required to determine MBCs for all of the isolates with the exception of one *A. popoffii* isolate (SC17) which had a MBC of 7.5 mg/L.

Discussion

One or more of the major components that comprised the essential oils evaluated in the present study, listed in Table 2, may have been responsible for the bactericidal effects to the *Aeromonas* isolates; however, specific single-variable tests would need to be done to identify which of the major components is primarily responsible for the antibacterial activities. Analyses showed that cinnamon oil (*C. cassia* = syn. *C. aromaticum*) is comprised primarily of cinnamaldehyde (61–99%), a component not present in the other oils we tested [19,20]. This suggests that cinnamaldehyde was responsible for the bactericidal effect by cinnamon oil to the *Aeromonas* spp., particularly to *A. salmonicida* subsp. *salmonicida*. According to the manufacturers of the cinnamon oils used in the present study (*C. cassia*; Lotus Brands Inc., Aromaland Inc.), their products typically contain between 65% and 82% cinnamaldehyde, values which are comparable to published values [20]. Similarly, carbacrol and alpha and beta citral, major components of oregano oil (*O. vulgare*) and lemongrass oil (*C. citratus*), respectively, are suspected to be the major components responsible for the bactericidal activities to *Aeromonas* spp. Analyses showed that oregano oil contains about 62% carvacrol [21] and according to the manufacturers, the oregano oils used in the present study contain between 65% and 82% carvacrol. Lemongrass oil contains a high concentration (80%) of alpha and beta citral [22]. The primary components of thyme oil (*T. vulgaris*) are thymol (40%) and *p*-cymene (18%); whereas rosemary (*R. officinalis*) is comprised of 44% *p*-cymene [23,24]. Based upon a comparison of the inhibition of *A. salmonicida* subsp. *salmonicida* by thyme oil and rosemary oil, the results presented in Table 2 suggest that the antibacterial efficacy was primarily associated with thymol rather than *p*-cymene. On the other hand, clove oil (*S. aromaticum*) and cinnamon oil (*C. zeylanicum*) contained high concentrations of eugenol (82–87%), yet moderately inhibited growth of *A. salmonicida* subsp. *salmonicida*. The primary components (linalool, manool, linalyl acetate, terpinen-4-ol) of other essential oils, namely, rosewood, sage, lavender and tea tree oil minimally inhibited *A. salmonicida* subsp. *salmonicida* in the disk diffusion susceptibility testing and MBC testing was not done. The antibacterial modes of action of essential oils and their major constituents are varied [25]. For instance, some major components of oils examined in the present study, including cinnamaldehyde, eugenol, thymol and linalool affect bacteria by permeabilizing membranes, inhibiting respiration, and altering membrane integrity that results in the release of cellular contents.

Similar to the bacterial inhibition results of the present study with the essential oils to *A. salmonicida* subsp. *salmonicida*, Bergonzelli et al. [15] showed that cinnamon oil, with cinnamaldehyde as the major component, was most inhibitory to growth of *Helicobacter pylori*. Inhibition of *H. pylori* by cinnamon oil was followed in descending order by inhibitions with oils of lemongrass, oregano and thyme. Bergonzelli et al. [15] also showed that clove oil had a moderate efficacy and oils of sage, tea tree, and basal had little if any inhibitory action to *H. pylori*.

Hammer et al. [7] determined the antimicrobial activity of fifty-two plant essential oils and plant extracts, including three essential oils used in the present study, to *Candida albicans* and a variety of bacteria including *A. veronii* bv. *sobria*, *Enterococcus faecalis*, *Escherichia coli* and *Staphylococcus aureus*. The range in minimum inhibitory concentrations (MIC's) with lemongrass oil to the organisms tested was 0.03–0.25% and the MIC for *A. veronii* bv. *sobria* was 0.12%. The MIC range for oregano oil was 0.12–2.0% (0.12% to *A. veronii* bv. *sobria*) and for thyme oil was 0.12% to greater than 2.0%, with a 0.12% MIC to *A. veronii* bv. *sobria*. Of all of the essential oils and plant extracts tested by Hammer et al. [7], MIC's of 0.12% were the lowest effective concentrations recorded for *A. veronii* bv. *sobria*. These results were comparable to the mean percent MBCs determined in the present study, shown in Table 5, for *A. veronii* bv. *sobria* obtained with lemongrass oil from Stony Mountain Botanicals (mean = 0.09%) and with oregano oils from Now Foods (0.10%) and Herbal Authority (0.13%). Furthermore, the ranges in mean percent MBCs in the present study for the three lemongrass oils (0.09–0.49%) did not statistically differ from one another. Similarly, mean MBCs (0.10–0.28%) with oregano oils from three commercial sources did not significantly differ from one another.

The sensitivities of *Aeromonas* spp. to lemongrass oil (*C. citratus*) reported by Singh et al. [26] were comparable to those of the isolates of the present study. Singh et al. [26] examined ninety-one *Aeromonas* isolates representing ten species and 78% (71/91) were sensitive to 50 µg/disk of lemongrass oil. In the present study, 79.7% (55/69) of the *Aeromonas* isolates were sensitive to less than 5% lemongrass oil from Stony Mountain Botanicals, and 85.5% (59/69) were sensitive to lemongrass oils from the other two commercial sources, Now Foods and Puritan's Pride. One contrast in the study by Singh et al. [26] and the present study was the sensitivity of *A. salmonicida* subsp. *salmonicida*. In the present study, all isolates were sensitive to lemongrass oils from all three sources with mean MBCs ranging from 0.11% to 0.31%, whereas three of five *A. salmonicida* subsp. *salmonicida* isolates were sensitive in the study by Singh et al. [26].

Certain essential oils may represent a promising alternative to antimicrobials for *Aeromonas* disease treatments in aquaculture. In host-challenge studies, certain essential oils that were incorporated in feed were effective in stimulating or enhancing immune function and reducing mortality. For example, two studies with tilapia (*Oreochromis officinalis* and *O. niloti-*

Table 5 Mean (±standard deviation) minimum bactericidal concentrations of essential oils and colloidal silver to *Aeromonas* spp.

Essential oil	*Aeromonas salmonicida* (10)[A]	*Aeromonas hydrophila* (5)	*Aeromonas veronii* bv. *sobria* (9)	*Aeromonas caviae* (1)	*Aeromonas popoffii* (17)	*Aeromonas allosaccharophila* (3)	*Aeromonas encheleia* (9)	*Aeromonas eucrenophila* (11)	*Aeromonas molluscorum* (4)
Cinnamon, Lotus	0.01 ± 0.01%^{a} [B]	0.03 ± 0.03%^{a}	0.02 ± 0.01%^{a}	0.04%	0.03 ± 0.02%^{a}	0.02 ± 0.01%^{a}	0.03 ± 0.02%^{a}	0.02 ± 0.02%^{a}	0.02 ± 0.01%^{a}
Cinnamon, Frontier	0.02 ± 0.01%^{a}	0.04 ± 0.03%^{a}	0.02 ± 0.01%^{a}	0.04%	0.04 ± 0.02%^{a}	0.03 ± 0.01%^{a}	0.05 ± 0.02%^{a}	0.03 ± 0.02%^{a}	0.03 ± 0.01%^{a}
Cinnamon, Aromaland	0.03 ± 0.01%^{a}	0.07 ± 0.05%^{a}	0.03 ± 0.02%^{a}	0.08%	0.03 ± 0.02%^{a}	0.04 ± 0.03%^{a}	0.02 ± 0.004%^{a}	0.08 ± 0.03%^{a}	0.06 ± 0.02%^{a}
Lemongrass, Stony Mountain Botanicals	0.11 ± 0.04%^{a}	0.13 ± 0.04% (3)[C]; > 5.0% (2)^{a}	0.09 ± 0.03% (8); > 5.0% (1)^{ab}	> 5.0%	0.08 ± 0.03% (15); > 5.0% (2)^{ab}	0.10 ± 0.04%^{a}	0.07 ± 0.02% (8); > 5.0% (1)^{a}	0.16 ± 0.00% (4); > 5.0% (7)^{abc}	0.16 ± 0.00% (3); > 5.0% (1)^{a}
Oregano, Now Foods	0.08 ± 0.00%^{a}	0.17 ± 0.11%^{a}	0.10 ± 0.04%^{ab}	0.16%	0.13 ± 0.08%^{ab}	0.10 ± 0.04%^{a}	0.16 ± 0.09%^{a}	0.22 ± 0.20%^{ab}	0.18 ± 0.09%^{a}
Oregano, Herbal Authority	0.08 ± 0.00%^{a}	0.23 ± 0.25% (4); > 0.67% (1)^{a}	0.13 ± 0.11%^{ab}	0.08%	0.12 ± 0.09% (14); > 0.67% (3)^{ab}	0.17 ± 0.12%^{a}	0.14 ± 0.09% (7); > 0.67% (2)^{a}	0.20 ± 0.19% (9); > 0.67% (2)^{ab}	0.35 ± 0.21%^{a}
Oregano, Stony Mountain Botanicals	0.17 ± 0.11%^{a}	0.37 ± 0.51%^{a}	0.28 ± 0.08%^{ab}	2.50%	0.16 ± 0.15% (16); > 5.0% (1)^{ab}	0.31 ± 0.00%^{a}	0.11 ± 0.08%^{a}	0.83 ± 0.47% (10); > 5% (1)^{bc}	0.31 ± 0.00%^{a}
Lemongrass, Now Foods	0.28 ± 0.06%^{a}	0.31 ± 0.00% (3); > 5.0% (2)^{a}	0.31 ± 0.00%^{ab}	0.31%	0.33 ± 0.10% (15); > 5.0% (2)^{ab}	0.31 ± 0.00%^{a}	0.66 ± 0.45% (8); > 5.0% (1)^{b}	0.31 ± 0.00% (8); > 5.0% (3)^{abc}	0.31 ± 0.00% (2); > 5.0% (2)^{a}
Lemongrass, Puritan's Pride	0.31 ± 0.00%^{a}	0.52 ± 0.15% (3); > 5.0% (2)^{a}	0.49 ± 0.30%^{b}	1.25%	0.67 ± 0.40% (15); > 5.0% (2)^{b}	0.63 ± 0.44%^{a}	0.78 ± 0.38% (8); > 5.0% (1)^{b}	1.02 ± 0.30% (8); > 5.0% (3)^{c}	0.94 ± 0.31% (2); > 5.0% (2)^{ab}
Thyme white	1.13 ± 0.34%^{b}	2.55 ± 1.98%^{b}	1.29 ± 0.55%^{c}	5.00%	2.33 ± 1.43% (16); > 5.0% (1)^{c}	1.67 ± 0.59%^{b}	1.81 ± 0.62%^{c}	2.88 ± 1.13% (10); > 5.0% (1)^{d}	3.13 ± 1.08%^{c}
Thyme linalol	2.29 ± 0.59%^{c}	0.31 ± 0.22% (3); > 5.0% (2)^{a}	2.23 ± 0.66% (8); > 5.0% (1)^{d}	> 5.0%	2.32 ± 0.63% (13); > 5.0% (4)^{c}	2.50 ± 0.00%^{c}	2.50 ± 0.00% (6); > 5.0% (3)^{d}	2.50 ± 0.00% (4); > 5.0% (7)^{d}	2.50% (1); > 5.0% (3)^{bc}
Allimed®-allicin, extract of garlic	> 5.0%	> 5.0%	> 5.0%	> 5.0%	5.0 ± 0.0% (7); > 5.0% (10)	> 5.0%	5.0 ± 0.0% (5); > 5.0% (4)	> 5.0%	> 5.0%
Colloidal silver	> 15.0 mg/L	> 15.0 mg/L	> 15.0 mg/L	> 15.0 mg/L	7.50 mg/L (1); > 15.0 mg/L (16)	> 15.0 mg/L	> 15.0 mg/L	> 15.0 mg/L	> 15.0 mg/L

[A] Number of isolates tested.

[B] Within each column, MBCs not followed by a common letter (a–d) significantly differ (Tukey–Kramer; $P < 0.05$). Allimed®, colloidal silver and *A. caviae* were not included in the analyses.

[C] Number of isolates that were not sensitive to the highest concentration of oil tested; if no number is provided, all isolates were included in the mean.

cus × *O. mossambicus*) showed reduced mortality in groups fed diets supplemented with extracts of *Rosmarinus officinalis* or cinnamon oil (*C. zeylanicum*) following challenges with *Streptococcus* spp. [27,28]. The highest concentration of Allimed® (5%) tested in the present study was bactericidal to seven *A. popoffii* and five *A. encheleia* isolates. In feeding studies, oven-dried garlic *A. sativum* was shown to significantly increase the survival of rohu *Labeo rohita* and rainbow trout *O. mykiss* due to infections with *A. hydrophila* [9,29]. Sahu et al. [29] fed a diet supplemented with garlic to rohu fingerlings for 60 days, then exposed them to 1.0×10^5 CFU/fish of *A. hydrophila* by IP injection. After 10 days, there was 85% and 71% survival among groups fed 0.10% and 1.0% garlic, respectively, compared to 57% survival in the control group. In a similar study, Nya and Austin [9] fed garlic to groups of rainbow trout fingerlings for 14 days prior to an IP injection challenge with 1×10^6 CFU *A. hydrophila* per fish. The fish were observed for 14 days with relative percent survivals of 91% for 0.10% garlic and 95% at the 1.0% level, compared with 88% mortality among control fish that were not fed garlic. The increased survival of the rohu and rainbow trout fingerlings to *A. hydrophila* afforded by the garlic was due to enhanced immune function [9,29]. It was shown in both of these studies that fish that were fed garlic demonstrated increased superoxide anion production, increased lysozyme and serum bactericidal activities, and greater serum total protein. Additionally, rainbow trout that were fed garlic had significantly increased growth and feed conversion, significantly higher phagocytic activity, hematocrits and respiratory burst, and increased numbers of erythrocytes and leukocytes [9].

The results of the present study along with those of previous studies suggests the need for further investigations into the potential use of essential oils to control fish diseases caused by *Aeromonas* spp., and perhaps other diseases caused by bacterial pathogens. To attain the maximum bactericidal effect, concerns such as effective treatment delivery methods for specific diseases, solubilities of essential oils in various water chemistries and potential host toxicity or unpalatability will need to be addressed and optimized.

Conclusion

Relatively low percent MBCs were achieved with essential oils, especially cinnamon, lemongrass and oregano to *Aeromonas* spp. *Aeromonas salmonicida* subsp. *salmonicida*, *A. hydrophila* and *A. veronii* bv. *sobria*, which are common pathogens to fishes, were particularly sensitive to these essential oils *in vitro*. The results obtained here warrant future experiments to evaluate potential disease treatments of affected hosts.

Conflict of interest statement

Any use of trade, product, or firm names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

Acknowledgements

The authors appreciate laboratory assistance provided by Pamela Whittington and Phyllis Randall. This article is the contribution 1807 of the US Geological Survey Great Lakes Science Center.

References

[1] Post G. Textbook of fish health. Neptune City, NJ: T.F.H. Publications, Inc.; 1987, p. 36.
[2] Winton JR. Fish health management. In: Wedemeyer GA, editor. Fish hatchery management. Bethesda, MD: American Fisheries Society; 2001. p. 559–639.
[3] Inglis V, Frerichs GN, Millar SD, Richards RH. Antibiotic resistance of *Aeromonas salmonicida* isolated from Atlantic salmon, *Salmo salar*, Scotland. J Fish Dis 1991;14(3):353–8.
[4] Alderman DJ, Hastings TS. Antibiotic use in aquaculture: development of antibiotic resistance-potential for consumer health risks. Int J Food Sci Technol 1998;33(2):139–55.
[5] Starliper CE, Cooper RK. Biochemical and conjugational studies of Romet-resistant strains of *Aeromonas salmonicida* from salmonid rearing facilities in the Eastern United States. J Aquat Anim Health 1998;10(3):221–9.
[6] Blazer VS, Iwanowicz LR, Starliper CE, Iwanowicz DD, Barbash P, Hedrick JD, et al. Mortality of centrarchid fishes in the Potomac drainage: survey results and overview of potential contributing factors. J Aquat Anim Health 2010;22(3):190–218.
[7] Hammer KA, Carson CF, Riley TV. Antimicrobial activity of essential oils and other plant extracts. J Appl Microbiol 1999;86(6):985–90.
[8] Nya EJ, Dawood Z, Austin B. The garlic component, allicin, prevents disease caused by *Aeromonas hydrophila* in rainbow trout, *Oncorhynchus mykiss* (Walbaum). J Fish Dis 2010;33(4):293–300.
[9] Nya EJ, Austin B. Use of garlic, *Allium sativum*, to control *Aeromonas hydrophila* infection in rainbow trout, *Oncorhynchus mykiss* (Walbaum). J Fish Dis 2009;32(11):963–70.
[10] Soltani M, Ghodratnema M, Ahari H, Ebrahimzadeh Mousavi HA, Atee M, Dastmalchi F, Rahmanya J. The inhibitory effect of silver nanoparticles on the bacterial fish pathogens, Streptococcus iniae, *Lactococcus garvieae*, *Yersinia ruckeri* and *Aeromonas hydrophila*. Int J Vet Res 2009;3(2):137–42.
[11] Koneman EW, Allen SD, Janda WM, Schreckenberger PC, Winn Jr WC. Color atlas and textbook of diagnostic microbiology. 4th ed. Philadelphia, PA: J.B. Lippincott Company; 1992, 1154pp.
[12] MacFaddin JF. Biochemical tests for identification of medical bacteria. 3rd ed. Philadelphia, PA: Lippincott Williams and Wilkins; 2000, 912pp.
[13] Miñana-Galbis D, Farfán M, Lorén JG, Fusté MC. Biochemical identification and numerical taxonomy of *Aeromonas* spp. isolated from environmental and clinical samples in Spain. J Appl Microbiol 2002;93(3):420–30.
[14] Beaz-Hidalgo R, Alperi A, Buján N, Romalde JL, Figueras MJ. Comparison of phenotypical and genetic identification of *Aeromonas* strains isolated from diseased fish. Syst Appl Microbiol 2010;33(3):149–53.
[15] Bergonzelli GE, Donnicola D, Porta N, Corthésy-Theulaz IE. Essential oils as components of a diet-based approach to management of *Helicobacter* infection. Antimicrob Agents Chemother 2003;47(10):3240–6.
[16] Petrus EM, Tinakumari S, Chai LC, Ubong A, Tunung R, Elexson N, Chai LF, Son R. A study on the minimum inhibitory concentration and minimum bactericidal concentration of nano-colloidal silver on food-borne pathogens. Int Food Res J 2011;18(1):55–66.
[17] Zar JH. Biostatistical analysis. 5th ed. Upper Saddle River, NJ: Prentice Hall; 2010, p. 226–32.

[18] Hintze J. NCSS 2001. Kaysville, UT: NCSS, LLC; 2001 <www.ncss.com>.
[19] Senanayake UM, Wijesekera ROB. Chemistry of Cinnamon and cassia. In: Ravindran PN, Nirmal Babu K, Shylaja M, editors. Cinnamon and cassia: the genus *Cinnamomum*. Boca Raton, FL: CRC Press; 2003. p. 80–120.
[20] Kong J-O, Lee S-M, Moon Y-S, Lee S-G, Ahn Y-J. Nematicidal activity of cassia and cinnamon oil compounds and related compounds toward *Bursaphelenchus xylophilus* (Nematoda: Parasitaphelenchidae). J Nematol 2007;39(1):31–6.
[21] Friedman M, Kozukue N, Harden LA. Cinnamaldehyde content in foods determined by gas chromatography–mass spectrometry. J Agric Food Chem 2000;48(11):5702–9.
[22] Leimann FV, Goncalves OH, Machado RAF, Bolzan A. Antimicrobial activity of microencapsulated lemongrass essential oil and the effect of experimental parameters on microcapsules size and morphology. Mater Sci Eng 2009;29(2):430–6.
[23] Albo GN, Henning C, Ringuelet J, Reynaldi FJ, De Giusti MR, Alippi AM. Evaluation of some essential oils for the control and prevention of American foulbrood disease in honey bees. Apidologie 2003;34(5):417–27.
[24] Özcan MM, Chalchat J-C. Chemical composition and antifungal activity of rosemary (*Rosmarinus officinalis* L.) oil from Turkey. Int J Food Sci Nutr 2008;59(7–8): 691–8.
[25] Hyldgaard M, Mygind T, Meyer RL. Essential oils in food preservation: mode of action, synergies, and interactions with food matrix components. Front Microbiol 2012;3(12):1–24.
[26] Singh BR, Singh V, Singh RK, Ebibeni N. Antimicrobial activity of lemongrass (*Cymbopogon citratus*) oil against microbes of environmental, clinical and food origin. Int Res Pharm Pharmacol 2011;1(9):228–36.
[27] Abutbul S, Golan-Goldhirsh A, Barazani O, Zilberg D. Use of *Rosmarinus officinalis* as a treatment against *Streptococcus iniae* in tilapia (*Oreochromis* sp.). Aquaculture 2004;238(1–4):97–105.
[28] Kanghear H, Suanyuk N, Khongpradit R, Subhadhirasakul S, Supamattaya K. Effect of cinnamon bark oil (*Cinnamomum zeylanicum* Blume) on the prevention of streptococcosis in sex-reversed red tilapia (*Oreochromis niloticus* × *O. mossambicus*). Songklanakarin. J Sci Technol 2005;27(Suppl. 1):347–58.
[29] Sahu S, Das BK, Mishra BK, Pradhan J, Sarangi N. Effect of *Allium sativum* on the immunity and survival of *Labeo rohita* infected with *Aeromonas hydrophila*. J Appl Ichthyol 2007;23(1):80–6.
[30] Alma MH, Ertas M, Nitz S, Kollmannsberger H. Chemical composition and content of essential oil from the bud of cultivated Turkish clove (*Syzygium aromaticum*). BioResources 2007;2(2):265–9.
[31] Mallavarapu GR, Ramesh S, Chandrasekhara RS, Rajeswara Rao BR, Kaul PN, Bhattacharya AK. Investigation of the essential oil of cinnamon leaf grown at Bangalore and Hyderabad. Flavour Fragr J 1995;10(4):239–42.
[32] Maia JGS, Andrade EHA, Couto HAR, da Silva ACM, Marx F, Henke C. Plant sources of Amazon rosewood oil. Quím Nova 2007;30(8):1906–10.
[33] Bernotiene G, Nivinskiene O, Butkiene R, Mockute D. Essential oil composition variability in sage (*Salvia officinalis* L.). Chemija 2007;18(4):38–43.
[34] Evandri MG, Battinelli L, Daniele C, Mastrangelo S, Bolle P, Mazzanti G. The antimutagenic activity of *Lavandula angustifolia* (lavender) essential oil in the bacterial reverse mutation assay. Food Chem Toxicol 2005;43(9):1381–7.
[35] Politeo O, Jukic M, Milos M. Chemical composition and antioxidant capacity of free volatile aglycones from basil (*Ocimum basilicum* L.) compared with its essential oil. Food Chem 2007;101(1):379–85.