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Comparative *in vitro* efficacy of eight essential oils as antibacterial agents against pathogenic bacteria isolated from pet-turtles

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ABSTRACT: Essential oils are plant extracts that have been used for their antimicrobial properties for centuries. The keeping of turtles as pets exhibits a growing trend worldwide but these animals are known to harbour a range of pathogenic bacteria. In the current study, we assessed eight essential oils as alternative antibacterial agents against nine species of pet turtle-borne Gram-negative bacteria, namely *Aeromonas caviae*, *A. dhakensis*, *A. hydrophila*, *Citrobacter freundii*, *Morganella morganii*, *Proteus mirabilis*, *P. vulgaris*, *Pseudomonas aeruginosa* and *Salmonella enterica*. Except for *Pseudomonas aeruginosa*, all other bacterial species showed high susceptibility to six essential oils, namely oregano, cinnamon, clove, lemongrass, lavender and eucalyptus oils in descending order of efficacy. Minimum inhibitory concentrations and minimum bactericidal concentrations values of the essential oils against all tested species except for *P. aeruginosa* showed low heterogeneity, showing that these essential oils can effectively control the growth of nearly all the tested. However, most of the tested bacteria were multiple-antibiotic-resistant as determined in the antibiotic disc diffusion test, with multiple-antibiotic-resistant index values of ≥ 0.2 for most of the strains. Therefore, with regards to their *in vitro* activity in controlling growth of multi-drug resistant bacteria, we can classify oregano, cinnamon, clove, lemongrass, lavender and eucalyptus essential oils as effective antibacterial agents. Thus, prospective application of these essential oils in controlling and treating these bacteria should be considered.

Keywords: essential oil; antibacterial efficacy; pet turtles; *Aeromonas caviae*; *Aeromonas dhakensis*; *Aeromonas hydrophila*; *Citrobacter freundii*; *Morganella morganii*; *Proteus mirabilis*; *Proteus vulgaris*; *Pseudomonas aeruginosa*; *Salmonella enterica;* cinnamon; *Cinnamomum zeylanicum*; clove; *Syzygium aromaticum*; eucalyptus; *Eucalyptus radiata*; ginger; *Zingiber officinale*; lemongrass; *Cymbopogon flexuosus*; lime; *Citrus aurantifolia*; lavender; *Lavandula angustifolia*; oregano; *Origanum vulgare*; yellow-bellied slider; *Trachemys scripta scripta*; Chinese stripe-necked turtle; *Ocadia sinensis*; river cooter; *Pseudemys concinna concinna*; Chinese softshell turtle; *Pelodiscus maackii*; western painted turtle; *Chrysemys picta belli*; common musk turtle; *Sternotherus odoratus*

Plant oils and extracts have been used for centuries, and these substances possess numerous biological properties (Jones 1996). The use of plant extracts as alternatives to chemical therapeutics has resulted in renewed attention in aromatic plants (Burt 2004). In particular, essential oils (EOs) have been tested as potential alternative remedies to treat many infectious diseases (Tepe et al. 2004). Generally, EOs are considered to be plant secondary metabolites and often possess antimicrobial and antioxidant properties (Hyldgaard et al. 2012). Since EOs are a rich source of biologically active compounds, investigating the antimicrobial properties of EOs extracted from aromatic plants is of growing interest (Hammer et al. 1999).

In line with the generally growing interest in exotic pet keeping worldwide, aquatic turtles are gaining in popularity as pets. Specifically, Republic

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of Korea is now among the top buyers of pet turtles from the USA (HSUS 2001). However, care must be taken when raising pet turtles, because they harbour numerous pathogenic bacteria in their normal flora which are either opportunistic or readily pathogenic to humans. Pet turtles have been reported to harbour a variety of antimicrobial-resistant bacteria such as *Aeromonas* spp., *Citrobacter freundii*, *Salmonella enterica*, *Edwardsiella tarda*, *Morganella morganii* and *Pseudomonas aeruginosa* in which resistance was often genetically determined (Diaz et al. 2006; Shin et al. 2016; Hossain et al. 2017; Wendt et al. 2017; Wimalasena et al. 2017a; Wimalasena et al. 2017b; Wimalasena et al. 2017c). Turtle-borne salmonellosis caused by *S. enterica* is often a concern in turtle-keeping (De Silva et al. 2017). *Aeromonas* spp. can cause gastroenteritis, wound and soft tissue infections, muscle infections, septicaemia and skin diseases (Janda and Abott 2010). *C. freundii* is known to cause opportunistic infections such as severe diarrhoea, urinary tract infections, pneumonia and brain abscesses (Badger et al. 1999). Besides, several opportunistic human infections caused by bacteria such as *E. tarda*, *M. morganii* and *P. aeruginosa* have been reported (Nelson et al. 2009; Bradbury et al. 2010; Liu et al. 2016).

Additionally, turtles can develop a range of bacterial zoonoses, especially when they are immunosuppressed. Necrotic stomatitis in turtles and tortoises has been determined to be caused by either *Pseudomonas* or *Aeromonas* bacteria (Holt et al. 1979). *C. freundii* has been identified as the main causative agent of septicaemic cutaneous ulcerative disease in aquatic turtles (Kobolkuti et al. 2008). *Aeromonas* and *Pseudomonas* spp. have been isolated from turtles with pneumonia, and many other infections in turtles with bacterial aetiologies have been described (McArthur 2004; Kohler 2006; Hernandez-Divers et al. 2009; Chen et al. 2013; Chung et al. 2017).

In the absence of vaccines against specific bacteria, antibiotics are common means of treating bacterial diseases or infections both in human and veterinary medicine. Nevertheless, indiscriminate and improper use of antibiotics has led to the global issue of antimicrobial resistance. Thus, investigating alternative medications for antibiotics is a matter of necessity, since conventional antimicrobial therapies might not be successful in treating such bacterial infections. Therefore, in the current study we sought to evaluate the *in vitro* efficacy of eight plant essential oils as antimicrobial agents against nine species of pathogenic Gram-negative bacteria isolated from pet turtles by comparative assessment of their minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs).

MATERIAL AND METHODS

Essential oils. Eight professional-grade EOs, namely cinnamon (*Cinnamomum zeylanicum*), clove (*Syzygium aromaticum*), eucalyptus (*Eucalyptus radiata*), ginger (*Zingiber officinale*), lemongrass (*Cymbopogon flexuosus*), lime (*Citrus aurantifolia*), lavender (*Lavandula angustifolia*) and oregano (*Origanum vulgare*), were purchased from Aromarant Co. Ltd., Rottingen, Germany. According to the manufacturer, the EOs has been extracted by steam distillation and 100% purity has been verified using a chiral method.

Bacteria. A total of 24 bacterial strains belonging to nine species, namely *A. caviae*, *A. dhakensis*, *A. hydrophilla*, *C. freundii*, *M. morganii*, *P. mirabilis*, *P. vulgaris*, *P. aeruginosa* and *S. enterica*, were selected for the study. Test strains had previously been isolated from six popular pet turtle species, namely the yellow-bellied slider (*Trachemys scripta scripta*), Chinese stripe-necked turtle (*Ocadia sinensis*), river cooter (*Pseudemys concinna concinna*), Chinese softshell turtle (*Pelodiscus maackii*), western painted turtle (*Chrysemys picta belli*) and common musk turtle (*Sternotherus odoratus*), which were reared in the laboratory in accordance with a general husbandry protocol (Bluvias and Eckert 2010). Species identity of all the test strains was confirmed using 16s rDNA sequencing and BLAST compatibility with the NCBI database.

Preliminary antibacterial assay. As is common, the disc diffusion test was used in preliminary examinations to screen the EOs for antibacterial activity and to select the most effective EOs (Burt 2004). All the test strains were cultured on tryptic soy agar (TSA) (MBcell, Los Angeles, USA) and incubated at 37 °C for 24 h prior to the test. Each bacterial inoculum was prepared in sterile saline to give a density equivalent to 0.5 McFarland (1.5×10^8 CFU/ml) units. Bacterial inocula were spread plated on Mueller-Hinton agar (MHA) (MBcell, Los Angeles, USA) using sterile cotton swabs in order to obtain evenly inoculated cultures. Under

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aseptic conditions, sterilised paper discs, 6 mm in diameter, (Advantec, Japan) were soaked with 20 µl of EOs at different concentrations (EO : dimethyl sulfoxide (DMSO) at 1 : 0, 1 : 1, 1 : 5 and 1 : 10) and placed on the agar surface (NCCLS 2002). Paper discs impregnated with DMSO were placed as the vehicle control. The plates were incubated at 37 °C for 24 h and the effectiveness of EOs was determined according to the inhibition zone diameters.

Minimum inhibitory concentrations and minimum bactericidal concentrations. The MICs of EOs were determined using the broth microdilution method (NCCLS 2002). Briefly, the 24-h TSA cultures were adjusted to approximately 1.5×10^8 CFU/ml with sterile saline solution. Culture medium was prepared by adding 5% (v/v) DMSO into double-strength Mueller Hinton broth and 100 µl of the medium were dispensed into each well of 96-well microtiter plates. For each EO, the first column of the well plate received EO with a final concentration of 4% (v/v) and then this initial solution was two-fold serially diluted across the plate until the concentration reached 0.0075% (v/v) based on the results of the preliminary antibacterial assay. One hundred microliters of each bacterial inoculum were added to wells and the plates were incubated at 37 °C for 24 h. Each assay was conducted in triplicate.

In order to determine the MBCs, the culture media from wells with EO concentrations higher than the MIC were smeared on TSA plates separately and incubated at 37 °C for 24 h. MBCs were determined as the lowest concentrations of EO which resulted in no viable bacterial colonies on the TSA plates.

Disc diffusion test for antibiotics. In parallel to the antibacterial assay for EOs, the susceptibility of 15 selected antibiotics from eight different antibiotic groups was examined on MHA following the recommendations of the Performance Standards for Antimicrobial Susceptibility Testing of the Clinical and Laboratory Standards Institute (CLSI 2014). The following antibiotics were tested: ampicillin (10 µg), amoxicillin (30 µg), cephalothin (30 µg), cefoxitin (30 µg), ceftriaxone (30 µg), imipenem (10 µg), meropenem (10 µg), gentamycin (10 µg), amikacin (30 µg), streptomycin (10 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), trimethoprim-sulfamethoxazole (25 µg), chloramphenicol (30 µg) and tetracycline (30 µg). The *E. coli* ATCC 25922 strain was employed in the antimicrobial susceptibility testing as the reference.

Multiple antibiotic resistance index. Following the disc diffusion test results for antibiotics, the multiple antibiotic resistance (MAR) index was calculated for each strain. When relating to a single bacterial strain, the MAR index is expressed as 'a/b', where 'a' equals the number of antibiotics to which the strain is resistant, and 'b' denotes the total number of antibiotics tested for the strain (Krumperman 1983).

RESULTS

According to the preliminary antibacterial assay, all EOs except for ginger oil and lime oil exhibited antibacterial activity against the tested bacteria (data not shown). The results revealed that those EOs showed antibacterial activities of varying magnitudes against the majority of the tested Gram-negative bacteria. Sensitivity was found to gradually increase with increasing concentration of EOs added to the disc. *P. aeruginosa* was observed to be susceptible only to oregano oil and cinnamon oil and was resistant against all other EOs at all the tested concentrations.

MIC and MBC test results for the six effective EOs (without ginger oil and lime oil) are given in Table 1 and Table 2. The MICs of ginger oil and lime oil were calculated to be > 17.8 mg/ml (> 4%) and > 17.2 mg/ml (> 4%), respectively, for all the tested strains. According to the MIC and MBC results for the other six EOs, the highest antibacterial activity (or the lowest MIC and MBC values) was detected in oregano oil, whereas the lowest efficacy was detected for eucalyptus oil.

The results of the antibiotic disc diffusion test are summarised in Table 3. With regards to the antibiogram profile, all tested bacteria, except for one *S. enterica* strain (MAR index 0.06), exhibited multiple drug resistance and showed resistance to three or more out of 15 antibiotics tested (MAR index ≥ 0.2). *P. aeruginosa* was the species that was resistant to the highest number of antibiotics, while *S. enterica* was resistant to the lowest number of antibiotics.

DISCUSSION

In the present study, we aimed to examine the *in vitro* efficacy of eight EOs in controling the growth of nine species of Gram-negative bacteria isolated

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Table 1. Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of the clove, oregano and cinnamon oils against pet turtle-borne pathogenic bacteria

Species	Strain	MIC and MBC of essential oils; mg/ml (% (v/v))								
		clove			oregano			cinnamon		
		MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC
Pseudomonas aeruginosa	PS1	> 21.98 (> 4)	ND	–	1.13 (0.25)	4.51 (1)	4	10.8 (2)	> 21.6 (> 4)	–
	PS2	> 21.98 (> 4)	ND	–	2.25 (0.5)	4.51 (1)	2	2.7 (0.5)	5.4 (1)	2
	PS3	> 21.98 (> 4)	ND	–	1.13 (0.25)	2.25 (0.5)	2	5.4 (1)	> 21.6 (> 4)	–
Proteus mirabilis	PM1	0.69 (0.125)	1.37 (0.25)	2	0.07 (0.015)	0.14 (0.03)	1	0.675 (0.125)	1.35 (0.25)	2
	PM2	1.37 (0.25)	2.75 (0.5)	2	0.07 (0.015)	0.14 (0.03)	1	0.675 (0.125)	2.7 (0.5)	4
	PM3	0.69 (0.125)	1.37 (0.25)	2	0.07 (0.015)	0.14 (0.03)	1	0.338 (0.06)	0.675 (0.125)	2
P. vulgaris	PV1	0.34 (0.06)	1.37 (0.25)	4	0.07 (0.015)	0.14 (0.03)	2	0.338 (0.06)	0.675 (0.125)	2
	PV2	0.34 (0.06)	1.37 (0.25)	4	0.14 (0.03)	0.14 (0.03)	1	0.675 (0.125)	0.675 (0.125)	1
	PV3	0.69 (0.125)	1.37 (0.25)	2	0.07 (0.015)	0.14 (0.03)	2	0.675 (0.125)	0.675 (0.125)	1
Morganella morganii	M1	1.37 (0.25)	2.75 (0.5)	2	0.035 (0.0075)	0.035 (0.0075)	1	0.675 (0.125)	0.675 (0.125)	1
	M2	0.69 (0.125)	1.37 (0.25)	2	0.035 (0.0075)	0.035 (0.0075)	1	0.675 (0.125)	0.675 (0.125)	1
	M3	0.69 (0.125)	1.37 (0.25)	2	0.07 (0.015)	0.07 (0.015)	1	0.675 (0.125)	1.35 (0.25)	2
Citrobacter freundii	CF1	0.69 (0.125)	0.69 (0.125)	1	0.07 (0.015)	0.07 (0.015)	1	0.338 (0.06)	0.675 (0.125)	2
	CF2	0.69 (0.125)	0.69 (0.125)	1	0.07 (0.015)	0.07 (0.015)	1	0.338 (0.06)	0.338 (0.06)	1
	CF3	0.69 (0.125)	0.69 (0.125)	1	0.07 (0.015)	0.07 (0.015)	1	0.338 (0.06)	0.675 (0.125)	2
Salmonella enterica	SE1	0.69 (0.125)	0.69 (0.125)	1	0.035 (0.0075)	0.035 (0.0075)	1	0.338 (0.06)	0.675 (0.125)	2
	SE2	0.34 (0.06)	0.34 (0.06)	1	0.035 (0.0075)	0.035 (0.0075)	1	0.675 (0.125)	0.675 (0.125)	1
	SE3	0.69 (0.125)	0.69 (0.125)	1	0.07 (0.015)	0.07 (0.015)	1	0.675 (0.125)	0.675 (0.125)	1
Aeromonas caviae	AC1	1.37 (0.25)	1.37 (0.25)	1	0.07 (0.015)	0.07 (0.015)	1	0.675 (0.125)	0.675 (0.125)	1
	AC2	1.37 (0.25)	1.37 (0.25)	1	0.07 (0.015)	0.07 (0.015)	1	0.675 (0.125)	1.35 (0.25)	2
A. dhakensis	AD1	2.75 (0.5)	2.75 (0.5)	1	0.07 (0.015)	0.56 (0.125)	1	1.35 (0.25)	1.35 (0.25)	1
	AD2	1.37 (0.25)	1.37 (0.25)	1	0.07 (0.015)	0.56 (0.125)	1	0.675 (0.125)	1.35 (0.25)	2
A. hydrophila	AH1	0.17 (0.03)	0.69 (0.125)	4	0.035 (0.0075)	0.07 (0.015)	2	0.338 (0.06)	0.338 (0.06)	1
	AH2	0.085 (0.015)	0.34 (0.06)	4	0.07 (0.015)	0.14 (0.03)	2	0.338 (0.06)	0.675 (0.125)	2
	AH3	0.34 (0.06)	0.69 (0.125)	2	0.14 (0.03)	0.14 (0.03)	1	1.35 (0.25)	2.7 (0.5)	2

ND = not done

from pet turtles. Six EOs were found to be suitable for this purpose. However, *P. aeruginosa* was highly tolerant to EOs compared to the other species, and only oregano and cinnamon oils could suppress its growth within the tested range of concentrations. As the MIC outcomes revealed, clove, eucalyptus, lemongrass, lavender, ginger and lime EOs all had MICs higher than 4% (v/v) against *P. aeruginosa*. Also, the MICs and MBCs of oregano and cinnamon oils against *P. aeruginosa* were comparatively much higher than those observed for the other bacterial species tested. Similar MIC values of eucalyptus, lime, lavender, clove and ginger oils of > 2% (v/v) were reported in *P. aeruginosa* (Hammer et al. 1999), while the cinnamon, clove and lime oil MICs were > 0.8, > 1.6 and > 6.4 (mg/ml), respectively (Prabuseenivasan et al. 2006). On the other hand, *P. aeruginosa* was resistant to at least 12 out of the 15 antibiotics tested and was only susceptible to ciprofloxacin towards which it exhibited the highest MAR index values of ≥ 0.86. Such resistance could be explained by the fact that *P. aeruginosa* is a unique pathogen known to employ all known bacterial resistance mechanisms such as chromosomal mutations, plasmid-mediated determinants, diminished outer membrane permeability, active efflux pump systems and various enzymatic modulators. Simultaneous employment of these mechanisms confers combined resistance to many antibacterial agents (Yordanov and Strateva 2009).

Among the tested EOs, oregano EO was found to be most effective, and the smallest MIC range,

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Table 2. Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of eucalyptus, lemongrass and lavender oils against pet turtle-borne pathogenic bacteria

Species	Strain	MIC and MBC of essential oils; mg/ml (% (v/v))								
		eucalyptus			lemongrass			lavender		
		MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC
Pseudomonas aeruginosa	PS1	> 18.14 (> 4)	ND	–	> 17.44 (> 4)	ND	–	> 18.5 (> 4)	ND	–
	PS2	> 18.14 (> 4)	ND	–	> 17.44 (> 4)	ND	–	> 18.5 (> 4)	ND	–
	PS3	> 18.14 (> 4)	ND	–	> 17.44 (> 4)	ND	–	> 18.5 (> 4)	ND	–
Proteus mirabilis	PM1	9.07 (2)	9.07 (2)	1	1.09 (0.25)	1.09 (0.25)	1	4.63 (1)	4.63 (1)	1
	PM2	9.07 (2)	> 18.14 (> 4)	–	0.55 (0.125)	1.09 (0.25)	2	4.63 (1)	4.63 (1)	1
	PM3	9.07 (2)	> 18.14 (> 4)	–	0.55 (0.125)	1.09 (0.25)	2	2.31 (0.5)	2.31 (0.5)	1
P. vulgaris	PV1	9.07 (2)	9.07 (2)	1	0.55 (0.125)	1.09 (0.25)	2	2.31 (0.5)	4.63 (1)	2
	PV2	9.07 (2)	> 18.14 (> 4)	–	0.27 (0.06)	1.09 (0.25)	2	4.63 (1)	4.63 (1)	1
	PV3	4.54 (1)	> 18.14 (> 4)	–	1.09 (0.25)	2.18 (0.5)	2	4.63 (1)	4.63 (1)	1
Morganella morganii	M1	4.54 (1)	4.54 (1)	1	0.27 (0.06)	0.55 (0.125)	2	2.31 (0.5)	4.63 (1)	2
	M2	4.54 (1)	4.54 (1)	1	0.27 (0.06)	0.27 (0.06)	1	4.63 (1)	4.63 (1)	1
	M3	2.27 (0.5)	4.54 (1)	2	0.27 (0.06)	0.55 (0.125)	2	2.31 (0.5)	2.31 (0.5)	1
Citrobacter freundii	CF1	4.54 (1)	4.54 (1)	1	0.55 (0.125)	0.55 (0.125)	1	2.31 (0.5)	9.25 (2)	4
	CF2	4.54 (1)	9.07 (2)	2	0.27 (0.06)	1.09 (0.25)	4	4.63 (1)	9.25 (2)	2
	CF3	9.07 (2)	9.07 (2)	1	0.27 (0.06)	0.55 (0.125)	2	4.63 (1)	9.25 (2)	2
Salmonella enterica	SE1	4.54 (1)	4.54 (1)	1	0.27 (0.06)	0.27 (0.06)	1	2.31 (0.5)	2.31 (0.5)	1
	SE2	4.54 (1)	9.07 (2)	2	0.55 (0.125)	0.55 (0.125)	1	4.63 (1)	4.63 (1)	1
	SE3	9.07 (2)	9.07 (2)	1	0.55 (0.125)	0.55 (0.125)	1	4.63 (1)	9.25 (2)	2
Aeromonas caviae	AC1	2.27 (0.5)	4.54 (1)	2	0.27 (0.06)	0.55 (0.125)	2	4.63 (1)	9.25 (2)	2
	AC2	2.27 (0.5)	4.54 (1)	2	0.27 (0.06)	0.55 (0.125)	2	4.63 (1)	9.25 (2)	2
A. dhakensis	AD1	4.54 (1)	9.07 (2)	2	2.18 (0.5)	4.36 (1)	2	4.63 (1)	9.25 (2)	2
	AD2	4.54 (1)	4.54 (1)	1	1.09 (0.25)	2.18 (0.5)	2	4.63 (1)	4.63 (1)	1
A. hydrophila	AH1	2.27 (0.5)	4.54 (1)	2	0.27 (0.06)	0.27 (0.06)	1	2.31 (0.5)	4.63 (1)	2
	AH2	2.27 (0.5)	4.54 (1)	2	0.14 (0.03)	0.14 (0.03)	1	4.63 (1)	4.63 (1)	1
	AH3	4.54 (1)	9.07 (2)	2	0.14 (0.03)	0.55 (0.125)	4	4.63 (1)	4.63 (1)	1

ND = not done

0.0075% (0.035 mg/ml) to 0.03% (0.14 mg/ml), was observed against the tested bacteria except for *P. aeruginosa*. Notably, oregano oil was effective in suppressing the growth of *P. aeruginosa* (MICs; 0.25% (1.13 mg/ml), 0.5% (0.25 mg/ml)). In addition, for oregano oil, MBC was equal to MIC in 18/24 (75%) of the isolates, which indicated the bactericidal effectiveness of oregano oil against all the tested species of bacteria. Antibacterial efficacy of oregano oil has been reported previously for foodborne *A. hydrophila*, *P. aeruginosa*, *S. enterica*, *P. mirabilis* and *P. vulgaris* (Souza et al. 2006; Ozkalp et al. 2010). The antibacterial efficacy of oregano oil could be attributed to carvacrol and thymol, the major phenolic compounds of oregano oil, which are capable of disintegrating the bacterial outer membrane, releasing lipopolysaccharides and increasing cell membrane permeability to permit ion loss from the cytoplasm (Ultee et al. 2002).

As the second most effective EO, cinnamon oil showed MIC values ranging from 0.06% to 0.25% (0.338–1.35 mg/ml), except for *P. aeruginosa*. The highest MIC, 0.25%, was observed for *A. dhakensis* and *A. hydrophila* while the other species had a similar MIC profile. Cinnamon oil has also been reported to restrain the growth of many Gram-positive and Gram-negative bacteria, some of which are multidrug-resistant (Keskin and Toroglu 2011; Guerra et al. 2012; Al-Mariri and Safi 2014). Cinnamaldehyde is the major component of cinnamon oil; it can exert different antibacterial activities depending on the concentration applied and is

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Table 3. Antibiogram profiles of pet turtle-borne pathogenic bacteria

Species	Strain	Antimicrobial susceptibility pattern			MAR index
		resistant	intermediate resistant	susceptible	
Pseudomonas aeruginosa	PS1	AMP, AMX, FOX, KF, STP, GEN, AK, IMI, MRP, NAL, SXT, CHL	CRO, TET	CIP	0.86
	PS2	AMP, AMX, FOX, KF, CRO, STP, GEN, AK, IMI, MRP, NAL, SXT, CHL, TET		CIP	0.93
	PS3	AMP, AMX, FOX, KF, STP, GEN, AK, IMI, MRP, NAL, SXT, CHL	CRO, TET	CIP	0.86
Proteus mirabilis	PM1	AMP, AMX, KF, SXT, GEN, CHL, TET	IMI, STP	FOX, CRO, MRP, AK, NAL, CIP	0.46
	PM2	AMP, TET, KF	STP	AMX, FOX, CRO, GEN, AK, IMI, MRP, NAL, CIP, SXT, CHL	0.2
	PM3	AMP, TET, FOX, KF	STP	AMX, CRO, GEN, AK, IMI, MRP, NAL, CIP, SXT, CHL	0.26
P. vulgaris	PV1	AMP, AMX, KF, STP	CHL	FOX, CRO, GEN, AK, IMI, MRP, NAL, CIP, SXT, TET	0.26
	PV2	AMP, TET, KF		AMX, FOX, CRO, STP, GEN, AK, IMI, MRP, NAL, CIP, SXT, CHL	0.2
	PV3	AMP, AMX, STP, KF, TET	GEN, CHL, IMI	FOX, CRO, AK, MRP, NAL, CIP, SXT	0.33
Morganella morganii	M1	AMP, AK, KF, FOX, IMI, SXT, TET	STP	AMX, CRO, GEN, MRP, NAL, CIP, CHL	0.46
	M2	AMP, AMX, KF, FOX, IMI, TET	SXT, MRP	CRO, STP, GEN, AK, NAL, CIP, CHL	0.4
	M3	AMP, AMX, KF, IMI	SXT	FOX, CRO, STP, GEN, AK, MRP, NAL, CIP, CHL, TET	0.26
Citrobacter freundii	CF1	AMP, AMX, KF, FOX, IMI, STP, NAL, CHL, TET	GEN	CRO, AK, MRP, CIP, SXT	0.6
	CF2	AMP, AMX, KF, FOX, STP, NAL, TET	SXT	CRO, IMI, MRP, GEN, AK, CIP, CHL	0.46
	CF3	AMP, AMX, KF, FOX, STP, GEN, NAL, SXT, CHL, TET	IMI, CIP	AK, MRP, CRO	0.66
Salmonella enterica	SE1	AMP, AMX, KF, CRO		FOX, IMI, MRP, STP, GEN, AK, NAL, CIP, SXT, CHL, TET	0.26
	SE2	AMP		AMX, KF, FOX, CRO, STP, GEN, AK, IMI, MRP, NAL, CIP, SXT, CHL, TET	0.06
	SE3	AMP, AMX, KF	CRO	FOX, IMI, MRP, STP, GEN, AK, NAL, CIP, SXT, CHL, TET	0.2
Aeromonas caviae	AC1	AMP, AMX, KF, GEN, SXT, CHL, TET	STP	FOX, CRO, AK, IMI, MRP, NAL, CIP	0.46
	AC2	AMP, AMX, KF, CHL	STP	FOX, CRO, IMI, MRP, GEN, AK, NAL, CIP, TET, SXT	0.26
A. dhakensis	AD1	AMP, AMX, KF, FOX, CRO, NAL, SXT, TET, CHL	CIP	STP, GEN, AK, IMI, MRP	0.6
	AD2	AMP, AMX, KF, FOX, CRO, STP, NAL, SXT, TET, CHL	CIP	IMI, MRP, GEN, AK	0.66
A. hydrophila	AH1	AMP, AMX, CRO, NAL, CHL	CIP, FOX	KF, STP, GEN, AK, IMI, MRP, SXT, TET	0.33
	AH2	AMP, AMX, KF, CRO, CHL, TET	FOX	STP, GEN, AK, IMI, MRP, NAL, CIP, SXT	0.4
	AH3	AMP, AMX, CRO, KF, NAL, CHL, TET	FOX, SXT	STP, GEN, AK, IMI, MRP, CIP	0.46

AK = amikacin, AMP = ampicillin, AMX = amoxicillin, CHL = chloramphenicol, CIP = ciprofloxacin, CRO = ceftriaxone, FOX = cefoxitin, GEN = gentamycin, IMI = imipenem, KF = cephalothin, MRP = meropenem, NAL = nalidixic acid, STP = streptomycin, SXT = trimethoprim-sulfamethoxazole, TET = tetracycline

capable of altering the lipid profile of the microbial cell membrane (Wendakoon and Sakaguchi 1995; Nazzaro et al. 2013). In the case of MBC, all the isolates except one *P. mirabilis* strain showed MBC/MIC values of ≤ 2 demonstrating the bactericidal effect of cinnamon oil. Generally, an agent can be

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considered bactericidal when the MBC/MIC ratio is < 4 and bacteriostatic when it is > 4 (Kone et al. 2004).

The MIC and MBC profile of clove oil was observed to be similar to cinnamon oil with the exception of *P. aeruginosa*. However, the lowest MIC of clove oil, obtained for one *A. hydrophila* isolate, was 0.03% (0.17 mg/ml), whereas the highest was 0.25% (1.37 mg/ml), which was found in a few other species. Also, the MBC/MIC values were relatively higher than those of cinnamon oil for most of the bacteria: against two *P. vulgaris* and two *A. hydrophila* isolates the ratio was 4. This indicates that clove oil could be bacteriostatic for these species. MICs > 3.2 and > 1.6 mg/ml have previously been reported for clove oil against *P. vulgaris* and *P. aeruginosa*, which are moderately higher than our results (Prabuseenivasan et al. 2006). Clove oil is rich in eugenol, a phenylpropene compound which has well-described antibacterial properties including alteration of cell membrane structure, the bacterial envelope and membrane-bound ATPase activity (Gill and Holley 2006).

When considering the lemongrass oil, the antibacterial activity was considerably effective at inhibiting the growth of all bacteria except *P. aeruginosa* with MIC values ranging from 0.03% (0.14 mg/ml) in *A. hydrophila* to 0.5% (2.18 mg/ml) in *A. dhakensis*. In addition, the activity of lemongrass oil against the majority of the tested isolates gave MICs ≤ 0.125% (0.55 mg/ml). In line with our results, a previous study reported MBC values of around 0.31% (v/v) in fish-borne *Aeromonas* spp. (Starliper et al. 2015). In contrast, lemongrass oil exerted only a minor effect on *C. freundii* and *P. mirabilis* and a lower number of isolates were found to be susceptible (Singh et al. 2011).

Although the relative efficacies of the eucalyptus and lavender oils were lower than those of other EOs, they could also inhibit the growth of all the tested Gram-negative bacteria except for *P. aeruginosa*. Lavender oil MICs were either 0.5% (2.31 mg/ml) or 1% (4.63 mg/ml), whereas eucalyptus oil showed MICs ranging from 0.5% (2.27 mg/ml) to 2% (9.07 mg/ml). In a previous study, the MICs of lavender oil for *Citrobacter* spp. and *Salmonella* spp. showed high values (> 10% (v/v)), although for *A. hydrophila* the MIC value was 0.94%. In the same study, eucalyptus oil was found to have MICs of 8.75%, 8.33% and 2.5% (v/v) for *Salmonella* spp., *Citrobacter* spp. and *A. hydrophila*, respectively (Mayaud et al. 2008). In addition, lime oil and ginger oil were relatively less effective with MICs of > 4% (v/v) for all of the isolates. This has been reported previously for several Gram-negative bacteria (Arora and Kaur 1999; Prabuseenivasan et al. 2006).

In the case of overall sensitivity to EOs, no species other than *P. aeruginosa* showed considerable heterogeneity in MIC and MBC values, which indicates that the EOs are effective against most of the tested bacteria regardless of the species. In addition, the MBC/MIC ratio was, on a whole, < 4 for EOs in the majority of the isolates demonstrating that the EOs are bactericidal for most of the strains. Most studies published to date were concerned with clinical, foodborne or bacteria from type culture collections. To our knowledge, ours is the first study to assess the EO sensitivity of Gram-negative bacteria isolated from turtles.

Meanwhile, the antibiotic resistance profile indicated that all the isolates excluding one *S. enterica* strain were resistant to three or more antibiotics with MAR index values of ≥ 0.2. Generally, MAR index of ≥ 0.20 implies that the bacteria originate from a site where several antibiotics have been frequently used and thus carry a high risk of contamination (Krumperman 1983). Importantly, in our study we could successfully deter the growth of such multidrug-resistant bacteria *in vitro* using EOs. Accordingly, the possible application of these EOs in controlling and treating infections of these Gram-negative bacteria should be considered.

In conclusion, the comparative efficacy of the eight EOs, in descending order, is oregano, cinnamon, clove, lemongrass, lavender, eucalyptus, ginger and lime. Our results on the *in vitro* efficacy of the eight EOs reveals oregano, cinnamon, clove and lemongrass oils to be the most effective alternative antibacterial agents against all the tested bacteria except for *P. aeruginosa*. However, further studies should be conducted to investigate the *in vivo* efficacy and other related parameters for a better elucidation of the potential of these EOs as practical antimicrobial agents.

REFERENCES

Al-Mariri A, Safi M (2014): In vitro antibacterial activity of several plant extracts and oils against some Gram-negative bacteria. Iranian Journal of Medical Science 39, 36–43.

https://doi.org/10.17221/142/2017-VETMED

Arora DS, Kaur J (1999): Antimicrobial activity of spices. International Journal of Antimicrobial Agents 12, 257–262.

Badger JL, Stins MF, Kim KS (1999): Citrobacter freundii invades and replicates in human brain microvascular endothelial cells. Infection and Immunity 67, 4208–4215.

Bluvias JE, Eckert KL (2010): Marine turtle trauma response procedures: a husbandry manual. Wider Caribbean Sea Turtle Conservation Network, USA. Technical report No. 10. 11–46.

Bradbury RS, Roddam LF, Merritt A, Reid DW, Champion AC (2010): Virulence gene distribution in clinical, nosocomial and environmental isolates of Pseudomonas aeruginosa. Journal of Medical Microbiology 59, 881–890.

Burt S (2004): Essential oils: their antibacterial properties and potential applications in foods-a review. International Journal of Food Microbiology 94, 223–253.

Chen J, Zhu N, Kong L, Bei Y, Zheng T, Ding X, He Z (2013): First case of soft shell disease in Chinese soft-shelled turtle (Trionyx sinens) associated with Aeromonas sobria-A. veronii complex. Aquaculture 406, 62–67.

Chung TH, Yi SW, Kim BS, Kim WI, Shin GW (2017): Identification and antibiotic resistance profiling of bacterial isolates from septicaemic soft-shelled turtles (Pelodiscus sinensis). Veterinarni Medicina 62, 169–177.

CLSI – Clinical and Laboratory Standards Institute (2014): Performance standards for antimicrobial susceptibility testing: Twenty-fourth informational supplement. CLSI M100-S24, Clinical and Laboratory Standards Institute, USA.

De Silva BCJ, Hossain S, Wimalasena SHMP, Pathirana HNKS, Wendt M, Heo GJ (2017): Quinolone susceptibility and genetic characterization of Salmonella enterica subsp. enterica isolated from pet turtles. Laboratory Animal Research 33, 49–56.

Diaz MA, Cooper RK, Cloeckaert A, Siebeling RJ (2006): Plasmid-mediated high-level gentamicin resistance among enteric bacteria isolated from pet turtles in Louisiana. Applied Environmental Microbiology 72, 306–312.

Gill AO, Holley RA (2006): Inhibition of membrane bound ATPases of Escherichia coli and Listeria monocytogenes by plant oil aromatics. International Journal of Food Microbiology 111, 170–174.

Guerra FQS, Mendes JM, Sousa JPD, Morais-Braga MFB, Santos BHC, Melo Coutinho HD, Lima EDO (2012): Increasing antibiotic activity against a multidrug-resistant Acinetobacter spp. by essential oils of Citrus limon and Cinnamomum zeylanicum. Natural Product Research 26, 2235–2238.

Hammer KA, Carson CF, Riley TV (1999): Antimicrobial activity of essential oils and other plant extracts. Journal of Applied Microbiology 86, 985–990.

Hernandez-Divers SJ, Hensel P, Gladden J, Hernandez-Divers SM, Buhlmann KA, Hagen C, Sanchez S, Latimer KS, Ard M, Camus AC (2009): Investigation of shell disease in map turtles (Graptemys spp.). Journal of Wildlife Diseases 45, 637–652.

Holt P, Cooper J, Needham J (1979): Diseases of tortoises: a review of seventy cases. Journal of Small Animal Practice 20, 269–286.

Hossain S, De Silva BCJ, Wimalasena SHMP, Pathirana HNKS, Heo GJ (2017): High prevalence of quinolone resistance genes in Citrobacter freundii isolated from pet turtles. Asian Journal of Animal and Veterinary Advances 12, 212–217.

HSUS – Humane Society of the United States (2001): The trade in live reptiles: exports from the United States. The Humane Society of the United States, USA.

Hyldgaard M, Mygind T, Meyer RL (2012): Essential oils in food preservation: Mode of action, synergies, and interactions with food matrix components. Frontiers in Microbiology 3, 12.

Janda JM, Abbott SL (2010): The genus Aeromonas: Taxonomy, pathogenicity, and infection. Clinical Microbiology Reviews 23, 35–73.

Jones FA (1996): Herbs-useful plants. Their role in history and today. European Journal of Gastroenterology and Hepatology 8, 1227–1231.

Keskin D, Toroglu S (2011): Studies on antimicrobial activities of solvent extracts of different spices. Journal of Environmental Biology 32, 251–256.

Kobolkuti LB, Czirjak G, Cadar D, Ungvari A, Uricaru A (2008): Septicemic/systemic cutaneous ulcerative diseases (SCUD) in captive red eared slider (Trachemys scripta elegans)-first report in Romania. Bulletin of USAMV Veterinary Medicine 65, 2.

Kohler G (ed.) (2006): Diseases of Amphibians and Reptiles. Krieger Publishing Company, Malabar. 184 pp.

Kone WM, Atindehou KK, Terreaux C, Hostettmann K, Traore D, Dosso M (2004): Traditional medicine in North Cote-d'Ivoire: screening of 50 medicinal plants for antibacterial activity. Journal of Ethnopharmacology 93, 43–49.

Krumperman PH (1983): Multiple antibiotic resistance indexing of Escherichia coli to identify high-risk sources of fecal contamination of foods. Applied Environmental Microbiology 46, 165–170.

Liu H, Zhu J, Hu Q, Rao X (2016): Morganella morganii, a non-negligent opportunistic pathogen. International Journal of Infection and Diseases 50, 10–17.

Mayaud L, Carricajo A, Zhiri A, Aubert G (2008): Comparison of bacteriostatic and bactericidal activity of 13 essential oils against strains with varying sensitivity to antibiotics. Letters in Applied Microbiology 47, 167–173.

https://doi.org/10.17221/142/2017-VETMED

McArthur S, Meyer J, Innis C (2004): Anatomy and physiology. In: McArthur S, Wilkinson R, Meyer J (eds): Medicine and Surgery of Tortoises and Turtles. Blackwell Publishing. 35–72.

Nazzaro F, Fratianni F, De Martino L, Coppola R, De Feo V (2013): Effect of essential oils on pathogenic bacteria. Pharmaceuticals 6, 1451–1474.

NCCLS – National Committee for Clinical Laboratory Standards (2002): Methods for dilution antimicrobial susceptibility tests of bacteria that grow aerobically. M100-S12, National Committee for Clinical Laboratory Standards, USA.

Nelson JJ, Nelson CA, Carter JE (2009): Extraintestinal manifestations of Edwardsiella tarda infection: a 10-year retrospective review. Journal of the Louisiana State Medical Society 161, 103–106.

Ozkalp B, Sevgi F, Ozcan M, Ozcan MM (2010): The antibacterial activity of essential oil of oregano (Origanum vulgare L.). Journal of Food, Agriculture and Environment 8, 272–274.

Prabuseenivasan S, Jayakumar M, Ignacimuthu S (2006): In vitro antibacterial activity of some plant essential oils. BMC Complementary and Alternative Medicine 6, 39.

Shin DM, Hossain S, Wimalasena S, Heo GJ (2016): Antimicrobial resistance and virulence factors of Edwardsiella tarda isolated from pet turtles. Pakistan Veterinary Journal 37, 85–89.

Singh BR, Singh V, Singh RK, Ebibeni N (2011): Antimicrobial activity of lemongrass (Cymbopogon citratus) oil against microbes of environmental, clinical and food origin. International Research Journal of Pharmacy and Pharmacology 1, 228–236.

Souza ELD, Stamford TLM, Lima EDO (2006): Sensitivity of spoiling and pathogen food-related bacteria to Origanum vulgare L. (Lamiaceae) essential oil. Brazilian Journal of Microbiology 37, 527–532.

Starliper CE, Ketola HG, Noyes AD, Schill WB, Henson FG, Chalupnicki MA, Dittman DE (2015): An investigation of the bactericidal activity of selected essential oils to Aeromonas spp. Journal of Advanced Research 6, 89–97.

Tepe B, Daferera D, Sokmen M, Polissiou M, Sokmen A (2004): In vitro antimicrobial and antioxidant activities of the essential oils and various extracts of Thymus eigii M. Zohary et P.H. Davis. Journal of Agricultural and Food Chemistry 52, 1132–1137.

Ultee A, Bennik MHJ, Moezelaar R (2002): The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen Bacillus cereus. Applied Environmental Microbiology 68, 1561–1568.

Wendakoon CN, Sakaguchi M (1995): Inhibition of amino acid decarboxylase activity of Enterobacter aerogenes by active components in spices. Journal of Food Protection 58, 280–283.

Wendt M, De Silva BCJ, Heo GJ (2017): Virulence factors and antimicrobial resistance of Pseudomonas aeruginosa isolated from pet turtles. Asian Journal of Animal and Veterinary Advances 12, 205–211.

Wimalasena SHMP, Shin GW, Pathirana HNKS, De Silva BCJ, Hossain S, Heo GJ (2017a): Characterization of quinolone resistant determinants in Morganella morganii isolated from pet turtles. Asian Journal of Animal and Veterinary Advances 12, 189–196.

Wimalasena SHMP, Shin GW, Hossain S, Heo GJ (2017b): Potential enterotoxicity and antimicrobial resistance pattern of Aeromonas species isolated from pet turtles and their environment. Journal of Veterinary Medical Science 79, 921–926.

Wimalasena SHMP, De Silva BCJ, Hossain S, Pathirana HNKS, Heo GJ (2017c): Prevalence and characterisation of quinolone resistance genes in Aeromonas spp. isolated from pet turtles in South Korea. Journal of Global Antimicrobial Resistance 11, 34–38.

Yordanov D, Strateva T (2009): Pseudomonas aeruginosa – a phenomenon of bacterial resistance. Journal of Medical Microbiology 58, 1133–1148.

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