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Antibacterial activity of essential oil from lavender (*Lavandula angustifolia*) against pet turtle-borne pathogenic bacteria

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Pet turtles are well-known to harbor an array of bacterial pathogens which can cause zoonotic infections in humans as well as opportunistic infections in the turtles itself. Essential oils are the natural plant extracts which have been traditionally used for disease treatment. In the present study, the essential oil of lavender (EOL) was examined for its antibacterial activity against thirty-eight strains of turtle-borne pathogenic bacteria belonging to seven species; *Aeromonas hydrophila, A. caviae, A. dhakensis, Citrobacter freundii, Proteus mirabilis, Salmonella enterica* and *Pseudomonas aeruginosa*. Antibacterial activity of EOL was tested by means of disk diffusion, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) tests. In addition, the antimicrobial susceptibility pattern of 11 commonly used antimicrobials was examined and the multiple antibiotic resistance (MAR) index was calculated. The results revealed that EOL was active against all tested turtle-borne pathogenic bacteria except *P. aeruginosa*. The range of MIC and MBC values of EOL against isolates except *P. aeruginosa* were recorded as 0.5-1% (V/V) and 0.5-2% (V/V), respectively. The MBC/MIC ratio was detected as <4, revealing that the tested EOL was bactericidal. Besides, most of the isolates were resistant to different antimicrobials in antimicrobial disk diffusion test. MAR index values of the tested strains were ranging from 0.27 to 0.91. The outcomes indicate that EOL has a potential to be used as an antibacterial agent against pathogenic bacteria isolated from pet turtles.

Keywords: Essential oil of lavender, antibacterial activity, pathogenic bacteria, pet turtles

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Synthetic antimicrobials and antibiotics have been used for a long time against different infectious diseases both in human and animals. The main drawback in antimicrobial chemotherapy is the growing antimicrobial resistance which makes the treatments less effective [1]. Therefore, the recent studies had given emphasize on the use of alternative natural products, especially which are obtained from plants [2,3].

Plants and plant extracts have been used as traditional medications for many centuries. Volatile compounds of plant extracts, particularly essential oils (EOs) are known as a secondary plant metabolite which had been used primarily in aromatherapy, cosmetics and medicinal purposes [4]. Various essential oils of different plants such as thyme, oregano, mint, cinnamon, cumin, salvia, clove, and eucalyptus have been observed to possess strong antimicrobial properties [5].

Essential oil of lavender (EOL) is known as one of the most popular essential oils which can be extracted from several lavender plant species. There are four major species of lavender namely; *Lavandula latifolia*, *Lavandula angustifolia*, *Lavandula stoechas* and *Lavandula x intermedia* (a sterile cross between *L. latifolia* and *L. angustifolia*) [6]. Among them, *Lavandula angustifolia* is the most extensively cultured species which is commonly recognized as commercial lavender. EOL is primarily composed of monoterpenoids and sesquiterpenoids where linalool and linalyl acetate are the most dominant.

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Lavandulyl acetate, terpinen-4-ol, and lavandulol are the medium level components while camphor and 1,8-cineole are contained in low to moderate in composition [7]. EOL itself and two major constituents of EOL, linalool and linalyl acetate are used in aromatherapy. Linalool and linalyl acetate have been detected as strong antimicrobial agents against foodborne bacteria such as *Escherichia coli* and *Enterobacter cloacae* [8]. Some other EOL compounds such as limonene, α-pinene, and β-pinene have antibacterial activity against different human pathogenic bacteria [9]. EOL was observed to have *in vitro* effect against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium* [10].

Turtles are popular reptilian species which are being used as pets worldwide. Meanwhile, pet turtles are known as a reservoir of different species of bacteria [11-13]. Antimicrobials are used for the treatment of different kinds of bacterial infection in pet turtle industry, but it caused the rise of resistance in their normal microflora [14]. Recently, the antimicrobial resistance determinants of several pet turtle-borne bacteria were investigated [15,16]. Pet turtles can transmit pathogenic bacteria by physical contact of turtles or contaminated cage environment if the pet owner is unaware of proper hygiene and sanitation. In the meantime, EOL has been recommended to treat lesions, conjunctivitis, constipation, skin and shell diseases of turtles and tortoises [17]. However, there were no previous reports about the antibacterial activity of EOL against turtle-borne bacteria.

Therefore, the present study was conducted to determine the antibacterial activity of EOL against pathogenic bacteria isolated from three popular pet turtle species through disk diffusion, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) tests for better understanding of the efficacy of EOL against turtle-borne bacteria.

Materials and Methods

Essential oil

EOL manufactured by EuroAroma®, Germany was purchased for the study. The EOL had been extracted from the flower of *Lavandula angustifolia* grown in Bulgaria and purified by steam distillation. The quality of the oil was determined as 100% pure.

Target organisms

In total, thirty-eight isolates belonging to seven bacterial species including 5 *Aeromonas hydrophila*, 3 *A. caviae*, 2 *A. dhakensis*, 11 *Citrobacter freundii,* 5 *Pseudomonas aeruginosa,* 6 *Salmonella enterica* and 6 *Proteus mirabilis* were isolated from three popular pet turtle species in Korea such as Chinese stripe-necked turtle (*Ocadia sinensis*), river cooter (*Pseudemys concinna concinna*) and yellow-bellied sliders (*Trachemys scripta scripta*) which were reared under laboratory condition.

Disk diffusion test

Disk diffusion test of EOL was performed using a 24 h cultured bacteria at 37ºC in tryptic soy agar (TSA) (MBcell Ltd., Seoul, Korea). The bacterial cultures were accustomed to 0.5 McFarland standard with sterile saline. The bacterial suspensions were spread over Mueller-Hinton agar (MHA) (MBcell Ltd., Seoul, Korea) plates using a sterile cotton swab. Different concentrations (1:1, 1:2, 1:4 and 1:10) of EOL were prepared using ethanol as the diluting agent. Sterilized paper discs (φ6 mm) were impregnated with 20 μL of different concentrations of EOL and placed on the inoculated agar plates. The plates were sealed with a sterile plastic wrap to avoid evaporation and incubated at 37ºC for 24 h. Finally, the diameter of inhibition zone was measured in mm.

Antimicrobial disks of the different antimicrobial group were used as controls against EOL. Amoxicillin (30 μg), ampicillin (10 μg), nalidixic acid (30 μg), amikacin (30 μg), cefoxitin (30 μg), ceftriaxone (30 μg), cephalothin (30 μg), ciprofloxacin (5 μg), imipenem (10 μg), gentamicin (10 μg), and streptomycin (10 μg) disks were used in antimicrobial disk diffusion test. The disk diffusion test and measurement of inhibition zone were executed according to the standards of the Clinical and Laboratory Standards Institute [18].

Multiple Antibiotic Resistance (MAR) index

Following the antimicrobial disk diffusion test results, one strain from each species showing the strongest resistance was selected for calculating the MAR index. MAR index was calculated as the ratio of number of resistant antimicrobials to total number of antimicrobials to which the strains were exposed.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) tests

The MIC test was performed by means of broth dilution method in a 96-well microtiter plate [19]. A portion of 100 μL of double strength Mueller-Hinton broth (MBcell Ltd., Seoul, Korea) containing 5% dimethyl sulfoxide (DMSO) (OCI Company Ltd, Seoul, Korea) was added to wells of 96-well microtiter plates. A series of two-fold dilution of EOL as 2, 1, 0.5, 0.25, 0.125, 0.063, 0.31, and 0.016% (V/V) was dispersed in the columns of wells. Finally, one hundred microliters (100 μL) of bacterial suspension prepared equivalent to 0.5 McFarland units with sterile saline was added to each well and incubated at 37°C for 24 h.

For observing MBC, 10 μL of the medium from wells with no visible growth was transferred to a TSA plate and incubated at 37°C for overnight. The MBC was determined as the lowest concentration that establishes a

Table 1. Antibacterial activity of EOL against pet turtle-borne bacteria

Isolate*	Inhibition zone[a] (mm) with different EOL concentrations				MIC (%) (V/V)	MBC (%)[b] (V/V)	MBC/MIC
	1:1	1:2	1:4	1:10			
AHy1	15	12	11	10	0.5	1	2
AHy2	9	8	7	7	1	1	1
AHy3	13.5	10.5	10	9.5	1	1	1
AHy4	12	9	7	6.5	1	2	2
AHy5	16	13	11	8	0.5	0.5	1
ACa1	11	10	8	7	1	1	1
ACa2	10	7	6.5	NA	1	1	1
ACa3	10	9	8	6.5	1	2	2
ADh1	25	15	14	11	0.5	1	2
ADh2	14	11	8	7	1	2	2
CFr1	9	8	7	NA	0.5	2	3
CFr2	9	7	NA	NA	0.5	1	2
CFr3	9	7	6.5	NA	0.5	2	3
CFr4	11.5	8	7	6.5	1	2	2
CFr5	11	9	7	6.5	1	2	2
CFr6	12	10	9	7	0.5	2	3
CFr7	9	7	6.5	NA	1	1	1
CFr8	11	9	8.5	7.5	0.5	2	3
CFr9	11	8	7	NA	1	2	2
CFr10	10	7.5	6.5	NA	1	2	2
CFr11	10	8	7.5	NA	0.5	0.5	1
PMi1	9	7.5	NA	NA	1	1	1
PMi2	11	NA	NA	NA	1	1	1
PMi3	10	NA	NA	NA	0.5	0.5	1
PMi4	9	NA	NA	NA	0.5	1	2
PMi5	9	6.5	NA	NA	1	1	1
PMi6	10	NA	NA	NA	1	1	1
SEn1	7	6.5	NA	NA	0.5	0.5	1
SEn2	8	6.5	NA	NA	1	1	1
SEn3	7	NA	NA	NA	1	2	2
SEn4	8	6.5	NA	NA	0.5	1	2
SEn5	9	7	7	6.5	1	2	2
SEn6	8	8	7.5	7	1	2	2
PAe1	NA	NA	NA	NA	>2	ND	ND
PAe2	NA	NA	NA	NA	>2	ND	ND
PAe3	NA	NA	NA	NA	>2	ND	ND
PAe4	NA	NA	NA	NA	>2	ND	ND
PAe5	NA	NA	NA	NA	>2	ND	ND

*Isolates: Bacterial strains of *A. hydrophila*, *A. carviae*, *A. dhakensis*, *C. freundii*, *P. mirabilis*, *S. enterica*, *P. aeruginosa* were designated as AHy, ACa, ADh, CFr, PMi, SEn and PAe, respectively.
[a]Inhibition zone: NA=No growth inhibition
[b]MBC: ND=not done

pre-determined reduction of bacteria (99.9%) in CFU/mL.

Results

Disk diffusion test

Disk diffusion test result of EOL against pet turtle-borne bacteria is shown in Table 1. According to the result, a similar pattern of antimicrobial activity was observed in all of the isolates except *P. aeruginosa*. All *P. aeruginosa* isolates were resistant even to the highest concentration of EOL. The highest activity of EOL was observed at 1:1 dilution for other bacterial isolates. Bacterial sensitivity to EOL was found in the pattern of

Table 2. Susceptibility patterns of turtle-borne bacteria against antimicrobials in disk diffusion test

Isolate*	Susceptibility patterns[a] against antimicrobials[b]											Total		
	AMP10	AMX30	FOX30	KF30	CRO30	IMI10	GEN10	CIP5	S10	NAL30	AK30	R	I	S
AHy1	R	R	S	S	S	S	S	R	S	S	S	3	0	8
AHy2	S	S	I	S	R	S	S	I	S	I	S	1	3	7
AHy3	R	R	S	R	S	S	S	S	S	S	S	3	0	8
AHy4	R	R	I	R	R	S	S	S	S	R	S	5	1	5
AHy5	R	R	S	S	S	S	S	S	S	S	S	2	0	9
ACa1	R	R	R	R	R	R	I	R	R	R	R	10	1	0
ACa2	R	R	S	R	S	S	S	R	S	S	S	4	0	7
ACa3	R	R	S	R	S	S	R	S	I	S	S	4	1	6
ADh1	R	S	S	S	S	S	S	R	S	I	S	2	8	1
ADh2	R	R	R	R	R	S	S	R	R	S	R	8	0	3
CFr1	R	R	R	R	S	S	I	R	R	R	S	7	1	3
CFr2	R	R	R	R	S	S	S	S	R	R	S	6	0	5
CFr3	R	R	R	R	S	S	S	S	S	R	S	6	0	6
CFr4	R	R	R	R	S	S	S	S	I	R	S	5	1	5
CFr5	R	R	R	R	S	S	S	S	S	R	S	5	0	6
CFr6	R	R	R	R	S	S	R	I	S	R	S	6	1	4
CFr7	R	R	R	R	S	S	S	I	S	R	S	5	1	5
CFr8	R	R	R	R	S	S	R	S	R	R	S	7	0	4
CFr9	R	R	R	R	S	I	R	I	R	R	S	7	2	2
CFr10	R	R	R	R	S	S	S	S	S	R	S	5	0	6
CFr11	R	R	R	R	S	S	S	S	S	R	S	5	0	6
PMi1	R	R	S	R	S	S	S	S	S	S	S	3	0	8
PMi2	S	S	S	S	S	S	S	S	S	S	S	0	0	11
PMi3	S	S	S	S	S	S	S	S	S	S	S	0	0	11
PMi4	S	S	S	S	S	S	S	S	S	S	S	0	0	11
PMi5	S	S	S	S	S	S	S	S	S	S	S	0	0	11
PMi6	S	S	S	S	S	S	S	S	S	S	S	0	0	11
SEn1	R	S	S	S	S	S	S	S	S	S	S	1	0	10
SEn2	S	S	S	S	S	S	S	S	S	S	S	0	0	11
SEn3	S	S	S	S	S	S	S	S	S	S	S	0	0	11
SEn4	S	S	S	S	S	S	S	S	S	S	S	0	0	11
SEn5	R	R	S	R	R	S	S	S	S	S	S	4	0	7
SEn6	R	R	R	R	S	S	S	S	S	I	S	4	1	6
PAe1	R	R	R	R	I	R	R	S	R	R	I	8	2	1
PAe2	R	R	R	R	I	R	R	R	R	R	I	9	2	0
PAe3	R	R	R	R	R	R	R	S	R	R	I	9	1	1
PAe4	R	R	R	R	R	R	S	S	R	R	R	9	0	2
PAe5	R	R	R	R	I	R	R	S	R	R	I	8	2	1

*Isolates: Bacterial strains of *A. hydrophila*, *A. carviae*, *A. dhakensis*, *C. freundii*, *P. mirabilis*, *S. enterica*, *P. aeruginosa* were designated as AHy, ACa, ADh, CFr, PMi, SEn and PAe, respectively.
[a]Susceptibility patterns: R=resistant, I=intermediate and S=susceptible were designated using breakpoint described by the Clinical Laboratory Standards Institute [18].
[b]Antimicrobials: AMP10=ampicillin (10 μg), AMX30=amoxicillin (30 μg), FOX30=cefoxitin (30 μg), KF30=cephalothin (30 μg), CRO30=Cefrtriaxone (30 μg), IMI10=Imipenem (10 μg), GEN10=gentamicin(10 μg), AK30=amikacin (30 μg), S10=Streptomycin (10 μg), NAL30=nalidixic acid (30 μg) and CIP5=ciprofloxacin (5 μg).

Table 3. MAR index value of antimicrobial resistance bacterial isolates used in this study

Bacterial species	No. of ineffective antimicrobials (total tested antimicrobials=11)	MAR value
Aeromonas hydrophila	5	0.45
Aeromonas caviae	10	0.91
Aeromonas dhakensis	8	0.73
Citrobacter freundii	7	0.64
Proteus mirabilis	3	0.27
Salmonella enterica	1	0.36
Pseudomonas aeruginosa	9	0.82

shrinking with the decreasing concentration. All *P. mirabilis* showed reduced susceptibility in 1:4 and 1:10 dilutions. Every *A. hydrpohila* and *A. dhakensis* isolates showed susceptibility against all tested concentrations of EOL. The maximum inhibition zone was observed in *A. dhakensis* with 25 mm. Four out of eleven *C. freundii* and two out of six *S. enterica* were sensitive even in 1:10 dilution.

According to the antimicrobial susceptibility patterns shown in Table 2, *P. aeruginosa* exhibited reduced susceptibility to all of the antimicrobials except ciprofloxacin and amikacin. *P. mirabilis* isolates showed susceptibility against every tested antimicrobial. All *C. freundii* showed resistance against at least five antimicrobials. With regards to *Aeromonas* spp., one *A. caviae* isolate was resistant to 10 antimicrobials except for gentamicin. Only two *S. enterica* strains were resistant to ampicillin, amoxicillin, cefoxitin, cephalothin and gentamicin.

MAR index

The highest value of MAR index was found in *A. caviae* which was 0.91 while the lowest MAR index was detected in *P. mirabilis* (0.27) (Table 3). MAR index of *P. aeruginosa*, *A. dhakensis*, *C. freundii A. hydrophila*, and *S. enterica* were 0.82, 0.73, 0.64, 0.45 and 0.36, respectively.

MIC and MBC

MIC test results of EOL against turtle-borne bacteria are shown in Table 1. Most of the strains were observed baring similar MICs ranged from 0.5 to 1% except *P. aeruginosa*. All *P. aeruginosa* strains showed the highest resistance against EOL (MIC>2%). The MIC was detected as 0.5% in 2 *A. hydrophila*, 1 *A. dhakensis*, 6 *C. freundii*, 2 *P. mirabilis* and 2 *S. enterica* strains. Additionally, 3 *A. caviae*, 1 *A. dhakensis,* 5 *C. freundii*, 4 *S. enterica*, 3 *A. hydrophila* and 4 *P. mirabilis* showed MIC as 1%.

MBC values, which were identical or greater than MIC are shown in Table 2. The highest ratio of MBC/ MIC was observed as 3 in only 4 *C. freundii* isolates. MBC/ MIC ratio of 2 was detected in most of the isolates including 2 *A. hydrophila*, 1 *A. caviae*, 1 *A. dhakensis*, 5 *C. freundii*, 1 *P. mirabilis*, and 4 *S. enterica*. MIC and MBC were identical in 3 *A. hydrophila*, 2 *A. caviae*, 2 *C. freundii*, 5 *P. mirabilis* and 2 *S. enterica*.

Discussion

Antimicrobials have been used for a long time to treat different bacterial infections, but the frequent use of these antimicrobials has resulted in growing antimicrobial resistance. Meanwhile, the essential oils have been shown strong antimicrobial activity against these pathogenic bacteria [20,21]. Therefore, the current study was conducted to determine the efficacy of EOL against pet turtle-borne bacteria.

In this study, the antimicrobial activity of EOL was investigated against pathogenic bacteria isolated from three popular pet turtle species. According to the disk diffusion test, all *A. hydrophila* and *A. dhakensis* showed susceptibility against every concentration of EOL. Some strains of *C. freundii* and *S. enterica* were sensitive even in the low concentrations. EOL was previously observed to inhibit the growth of *A. hydrophila*, *C. freundii* and *S. enterica* strains in disk diffusion test [22]. Similarly, another study also encountered EOL susceptible *P. mirabilis* [8]. EOL had shown mostly strong antibacterial activity against Gram-positive bacteria and EOL had also been effective against Gram-negative bacteria [23, 24]. In addition, EOL was found to be effective against some multi-drug resistant clinical Gram-negative bacteria such as *E. coli* and *Acinetobacter baummanii* [2,5].

All tested *P. aeruginosa* strains were resistant to EOL both in disk diffusion and MIC tests while showing reduced susceptibility to 81% of tested antimicrobials. A similar result was found in a recent study where *P. aeruginosa* was resistant to EOL (MIC>5%) [25]. *P. aeruginosa* is an opportunistic pathogen which often showed resistance against antimicrobials and EO of plants [26]. Such resistance pattern of *P. aeruginosa* could be the result of an outer-membrane which is predominantly impervious to phenolic compounds of

EO, the existence of efflux mechanisms and porine-related inhibition which are protecting the bacteria against the action of EO [27].

It could be noted that the tested strains of our study which were sensitive to EOL showed resistance to antimicrobials such as penicillin, cephalosporin, aminoglycoside and quinolone groups. Each of the bacterial species displayed high MAR index ranged from 0.27-0.91 which is greater than 0.2. The higher level of MAR index value (0.2) indicates that the bacteria survived from a high-risk source of contamination where the antimicrobials had been frequently used [28].

In accordance with MIC and MBC results, all of the isolates had MIC ranged from 0.5 to 1%. Among them, the majority of the isolates showed MIC 1%. In a different study, clinical *Salmonella* spp. and *Citrobacter* spp. showed a high value of MIC against EOL (MIC >10%). In the same study, *A. hydrophila* exhibited MIC value of 8±0.94% [29]. In our study, MBC/MIC ratio was estimated to examine the antimicrobial activity of EOL. The efficacy of an antimicrobial agent is dependent on MIC and MBC values and their inter-relationship [30]. An antimicrobial agent is regarded as bactericidal if the MBC value is not more than 4 times of the MIC value [31]. In the present study, all of the isolates showed MBC/MIC as 4 against EOL revealing that EOL was able to kill all of the isolates except *P. aeruginosa*. The high antibacterial activity of EOL against turtle-borne bacteria was observed which could be resulted due to the antimicrobial activity of the EOL constituents. According to the previous report, the presence of high amount of active phenolic compounds in EOL such as linalool and linalyl acetate exhibited strong antimicrobial properties [23]. The major phenolic compounds of EOL vary with different origin of lavender samples. A previous study reported that EOL of Bulgarian origin (51.9% linalool, 9.5% linalyl acetate) was more effective than EOL of French origin (43.2% linalool, 29.1% linalyl acetate) against 25 bacterial species [32]. In another study, EOL of Bulgarian origin had the highest antibacterial activity than EOL of other origins [22]. Since EOL originated in Bulgaria was used in the present study, it could be the reason EOL to be effective against most of the bacteria.

According to the findings, it can be concluded that EOL is effective against most of the turtle-borne pathogenic bacteria. Hence, EOL could be a good choice to control different bacterial infections of pet turtles. On the other hand, the high MAR index values of most of the bacterial strains should not be underestimated because of the public health concern. Therefore, further study should be focused on determining the efficacy of other essential oils against pathogenic bacteria isolated from pet turtles.

Conflict of interests The authors declare that there is no financial conflict of interests to publish these results.

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