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Original Article

Antimicrobial properties of *Thymus vulgaris*, *Origanum majorana* and *Ziziphora clinopodioides* combined essential oils and their effects on growth behavior of *Aeromonas hydrophila*

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Abstract: This study aimed to determine the chemical composition of *Thymus vulgaris*, *Origanum majorana* and *Ziziphora clinopodioides* and evaluate the antimicrobial properties of their combined essential oils i.e. T.o.z 50% T.v, 25% O.m and 25% Z.c; t.O.z = 25% T.v, 50% O.m and 25% Z.c and t.o.Z = 25% T.v, 25% O.m and 50% Z.c, against *Aeromonas Hydrophila* (in vitro). The compositions of the herbal essential oils were determined using gas chromatography–mass spectrometry (GC-MS) and the antimicrobial effects was conducted using agar-disc diffusion method, determination of MIC and MBC, and bacterial growth curves determination based on OD at 600 nm. The main compounds were Thymol (40.60%) and Limonene (15.98%) for *T. vulgaris*, Carvacrol (57.86%) and Thymol (13.54%) as the major compounds in *O. magorana*. Regarding *Z. clinopodiodes*, α-pinene (22.6%) and Carvacrol (21.1%) represented the major constituents. Base on the disc-diffusion results, t.O.z showed the best inhibition zone (26 mm). The inhibitory activity and bactericidal effect of t.O.z, unveiled by the MIC and MBC values, was clearly the highest between all combined herbal oils. Regarding the bacterial growth curves, the combined essential oils exhibited significant differences in all tested concentrations. The t.O.z was the most effective between the three tested combined oils at different temperature that cannot affect herbal oil performance. As conclusion, we suggested that the mixing of herbal oils with growth media was delaying exponential phase starting in the bacterial growth curves.

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Introduction

Application of antibiotics has been led to increase in bacterial resistance, and therefore, scientists has recently focused on herbal extracts as a natural antioxidants and antibacterial substances (Dhull et al., 2016). In addition, herbal extracts have been reported to promote various functions such as growth, appetite stimulation, stress resistance, immune functions, skin coloration, egg hatching rates, hematological and biochemical status as well as increasing disease resistance in aquaculture due to having different active components (Yılmaz et al., 2010). The antimicrobial properties of medicinal plant's essential oils are due to their hydrophobic characteristic that act over the lipids of the cell membrane, modifying its structure and turning it more permeable, and allowing the passage of ions and or other substances (Millezi et al., 2012). According to Ouedrhiri et al. (2016) essential oils are also accumulated in the cytoplasmic membrane, causing damages such as loss of function of selective barrier.

Thyme, *Thymus vulgaris*, an aromatic plant of the Lamiaceae family, is typical of the Mediterranean area and extensively used as a culinary herb. Its essential oil is utilized as flavor ingredients in a wide variety of food, beverage and confectionery products, as well as in perfumery for the scenting of soaps and lotions. Because of its antiseptic, antispasmodic and antimicrobial properties, it is also used for medicinal purposes (Cosentino et al., 1999). Thyme has a strong antimicrobial and antioxidant activity due to its very high contents of Thymol, P-Cymene, Carvacrol, Eugenol, and 4-allylphenol (Gültepe et al., 2014). The antioxidative effect of thyme is based on polyphenolic

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compounds as flavonoids, while Thymol and Carvacrol have lower activity (Selmi and Sadok, 2008).

Marjoram, *Origanum majorana*, belongs to the Lamiaceae family with interesting pharmacological effects (Dantas et al., 2016). It is an aromatic plant rich in essential oils and commercially grown in southern Europe and in the Mediterranean region (El-Ashmawy et al., 2007; Ramadan et al., 2012). Marjoram has an extensive range of biological activity, such as antioxidant, antimicrobial, anti-inflammatory, antitumorigenic and hepatoprotective activities (Abdel-Massih et al., 2010; Al Dhaheri et al., 2013).

Ziziphora clinopodioides, a member of the family Lamiaceae, grows in several regions throughout the world, especially Middle East (Tian et al., 2011; Shahbazi, 2017). Its antibacterial, antioxidant, antifungal, anti-inflammatory properties is already reported (Tian et al., 2010; Kheirkhah et al., 2015). Moreover, its essential oil has been proved to possess insecticidal activity (Lolestani and Shayesteh, 2009).

Aeromonas hydrophila is a motile, facultatively anaerobic Gram-negative rod, oxidase and catalase-positive bacterium, participating with other members of this genus in Motile Aeromonad Infection (MAI) which is probably the most common bacterial disease of freshwater fishes (Cipriano et al., 1984). This motile aeromonad may also inhabits brackish water but decrease in prevalence with increasing salinity (Thune et al., 1993). Motile aeromonads are relatively weak pathogens but isolate widely in pathogenicity. An S-layer on the cell wall and more elastase production are present in more pathogenic strains. While both endotoxin and exotoxins (proteases, hemolysins) are produced, their precise relationship with pathogenicity is unclear (Peatman et al., 2018). Some references consider motile aeromonads as a part of the normal intestinal microflora of healthy fish and disease occurrence are usually secondary to infection by a primary pathogen (Cipriano et al., 1984). The in-vitro conditions in which microorganisms can be able to grow such as temperature and pH are particularly simulated those in certain parts of the host body (for instance, respiratory system, gastrointestinal and urogenital tracts) (Vukanti et al., 2012). Therefore, this research aimed to evaluate the antibacterial combined effects of essential oils extracted from *Thymus vulgaris*, *O. Majorana* and *Z. clinopodioides* on optimal inhibitory effect against *A. hydrophila* in different temperatures.

Materials and Methods

Microorganisms: *Aeromonas hydrophila* was obtained from a bacterial bank in Department of Aquatic Animal Health and Disease - University of Tehran. Bacterial samples were cultured on blood-agar media and overnight-cultures were used in the conducted experiments.

Essential Oils: *Thymus vulgaris, O. majorana* and *Z. clinopodioides* essential oils were provided from a commercial firm (Pars-Imen daroo, Iran). Three combination of the essential oils (CEO) were prepared as follows: T.o.z contains 50% *T. vulgaris*, 25% *O. majorana* and 25% *Z. clinopodioides*; t.O.z = 25% *T. vulgaris,* 50% *O. majorana* and 25% *Z. clinopodioides* and t.o.Z = 25% *T. vulgaris,* 25% *O. majorana* and 50% *Z. clinopodioides*. The compositions of the herbal essential oils in this study were determined by gas chromatography–mass spectrometry (GC-MS). The analyses were performed using Shimadzu GC2010 system with an auto injector AOC-20i and Plus mass detector QP2110, equipped with an HP5-MS fused silica capillary column (30 m × 0.25 mm × 0.25 μm). The chromatographic conditions were as follows: carrier gas, helium at a flow rate of 1.02 mL min^{-1}; oven temperature programmed initially at 60°C and increased to 310°C at a ramp of 3°C min^{-1}; injector temperature, 220°C; injector mode in split ratio of 1:20 with 2 mL min^{-1} purge; MS interface temperature, 280°C; ion source temperature, 260°C; and ionization energy, 70 eV. The oil samples (15 mg) were dissolved in 1.5 mL of purified ethyl acetate and 1 μL this solution was injected for analysis. The isolated compounds were identified by their respective Kováts retention indices determined in reference to a series of n-alkanes, and

verified by a comparison of mass spectral data with those obtained using pure standards and those reported in the literature, 10 and eventually by comparing their mass spectra with the GC–MS spectral library (Wiley 8 and FFNSC 1.2 libraries).

Antibacterial assays

Agar-disc diffusion method: The essential oils were first screened for their antibacterial activity against *A. hydrophila* using agar-disc diffusion method according to CLSI (2012) with some modifications. Briefly, fresh bacterial suspension was prepared in physiological solution (sodium chloride 0.9%) and adjusted to (1) McFarland used to inoculate on Agar plates (TSA-Merck, Germany). Then, the sterile paper discs (6.4 mm in diameter) were applied on the surface of each plate and impregnated with 10 µL of essential oil. The plates were incubated at 25ºC for 18-24 h. The diameters of inhibition zones were measured in mm. Florfenicol, Oxytetracycline, Trimethoprim-sulfamethoxazole and Enrofloxacin were used as positive samples and all the experiments were carried out in triplicate.

Micro-well determination of MIC: The minimum inhibition concentration (MIC) values were determined for the three CEOs as described by Hajlaoui et al. (2016). The inoculums of *A. hydrophila* strain were prepared from 24 h broth cultures, and suspensions were adjusted to (0.5) McFarland standard turbidity. T.o.z, t.O.z and t.o.Z were dissolved in 10% dimethyl sulfoxide DMSO (Sigma-Aldrich) and diluted to the highest concentrations (12.8 µL /mL). Then serial two-fold dilutions were prepared i.e. concentrations of 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4 and 12.8 µL /mL from each CEO into 5 ml sterile test tubes containing nutrient broth. The 96-well plates were prepared by dispensing 95 µL of nutrient broth and 5 µL of the bacterial inoculum into each well. A 100 µL aliquot from the stock solutions of the combined essential oil was added into the first wells. Then, 100 µL from the serial dilutions were transferred into the consecutive wells. The last well containing 95 µL of nutrient broth without CEO and 5 µL of the bacterial inoculum on each strip was used as the negative control. The final volume in each well was 100 µL. The plates were incubated at 25°C for 18-24 h. The experiments were carried out in triplicate for each CEO concentration. After 18-24 h, the results were taken by ELISA microplate reader (OD at 600 nm) and the MIC value was defined as the lowest concentration (the highest dilution) of the compounds to inhibit the growth of the microorganisms.

Determination of (MBC): The minimum bactericidal concentrations were determined by spreading 5 µL from negative wells in the previous stage (i.e. MIC determination) on blood agar plates. The MBC value corresponded to the lowest concentration of the combined essential oil yielding negative subculture after incubation at 25ºC for 24 h (Ouedrhiri et al., 2016).

Bacterial growth curves: Bacterial growth intensities were determined in 96-well plates with assay similar to that one in MIC determination. For every combined essential oil, four concentrations (lower than MBC concentration) were used to investigate their effects on *A. hydrophila* growth curves. This trail was conducted in different temperature of 20, 25 and 30°C, PH=7 which was monitored by titration with 5% H_2SO_4 or 5% NaOH. 96-well plates were incubated in a shaker cold incubator (FG-Iran). Growth curves were obtained by measuring the optical density (OD) at 600 nm (by ELISA microplate reader) at regular time intervals (1, 3, 6, 9, 12, 24, 48, 96 h). All experiments were conducted for 5 replicates and resulting OD data over time for each replicate-sample was analyzed for growth yield (maximum absorbance at 600 nm) (Vukanti et al., 2012).

Statistical analyses: Data for each variable at each time point were compared using ANOVA method and Duncan test in SPSS var.17, and significance was determined if P-value is less than 0.05 (n = 5).

Results

Chemical composition of essential oils: A total of 18 components were determined accounting for 99.42% of the total amount of *T. vulgaris* essential oil (Table 1). The main compounds were Thymol (40.60%),

Table 1. Chemical composition of *Thymus vulgaris*, *Origanum majorana* and *Ziziphora clinopodioides* essential oils.

	T. vulgaris		***O. majorana***		***Z. clinopodioides***	
compound	**RT (min)**	**Area (%)**	**RT (min)**	**Area (%)**	**RT (min)**	**Area (%)**
α-Pinene	6.708	0.46	6.158	0.17	6.631	**22.6**
Camphene	-	-	-	-	7.077	1.6
β-Pinene	8.255	0.96	-	-	8.078	0.8
β-Myrcene	8.836	0.66	8.191	0.10	8.654	0.3
Camphane	9.131	0.53	-	-	8.935	0.2
δ.3-Carene	9.588	0.67	-	-	9.391	0.5
Isocineole	9.811	1.93	-	-	9.599	3.6
1-Phellandrene	9.375	2.88	-	-	-	-
α-Terpinene	9.899	3.22	9.177	0.09	-	-
Cymene	10.309	**12.17**	9.578	6.78	-	-
γ-Terpinene	11.752	5.82	10.99	3.11	-	-
Dihydrocarvone	18.108	0.18	-	-	-	-
Trans-Caryophyllene	27.971	0.32	27.366	**11.52**	-	-
Cymol	-	-	-	-	10.035	8.2
Limonene	10.548	**15.98**	9.696	0.67	10.216	8.3
1.8-Cineole	-	-	-	-	10.315	9.1
α-Terpinolene	13.08	8.90	-	-	12.753	0.8
Linalool L.	-	-	-	-	13.314	0.5
D-Fenchyl alcohol	14.133	0.16	-	-	13.853	0.3
Terpinene 1-OL	-	-	-	-	14.818	0.3
Cyclohexanone, 5-	-	-	-	-	15.695	3.0
Ethanone, 1-	-	-	-	-	16.214	1.1
Menthol	-	-	-	-	16.619	3.4
α-Thujene	-	-	5.942	0.08	-	-
2-δ-pinene	-	-	7.616	0.61	-	-
α-Cubebene	-	-	24.547	0.10	-	-
3-Allyl-6-	-	-	25.097	1.98	-	-
Copaene	-	-	25.579	0.43	-	-
α-Humulene	-	-	28.481	1.57	-	-
δ-Cadinene	-	-	30.9	0.17	-	-
4-Terpineol	-	-	-	-	16.775	0.3
Para-Cymen-8-ol	-	-	-	-	17.184	0.9
α-Terpineol	-	-	-	-	17.408	0.8
Pulegone	-	-	-	-	19.867	0.6
Carvone	20.666	3.03	-	-	20.132	3.3
Anethole	22.529	0.94	-		22.249	0.5
Thymol	23.468	**40.60**	22.56	**13.54**	22.778	7.0
Carvacrol	-	-	23.556	**57.86**	23.292	**21.1**
Piperitenone	-	-	-	-	24.666	0.6
Caryophyllene oxide	-	-	32.758	0.27	33.233	0.4
Total	-	99.42	-	99.06	-	100.0

Limonene (15.98%), Cymene (12.17%) and α-Terpinolene (8.90%). Other compounds were found in small quantities ranging between 0.16% (D-Fenchyl alcohol) to 5.82% (γ-Terpinene). In the *O. majorana*

Table 2. Inhibitory and bactericidal effects against *Aeromonas hydrophila* (values= µL /mL).

Combined oil	concentration							
	0.1	0.2	0.4	0.8	1.6	3.2	6.4	12.8
T.o.z				MIC		MBC		
t.O.z			MIC			MBC		
t.o.Z				MIC		MBC		

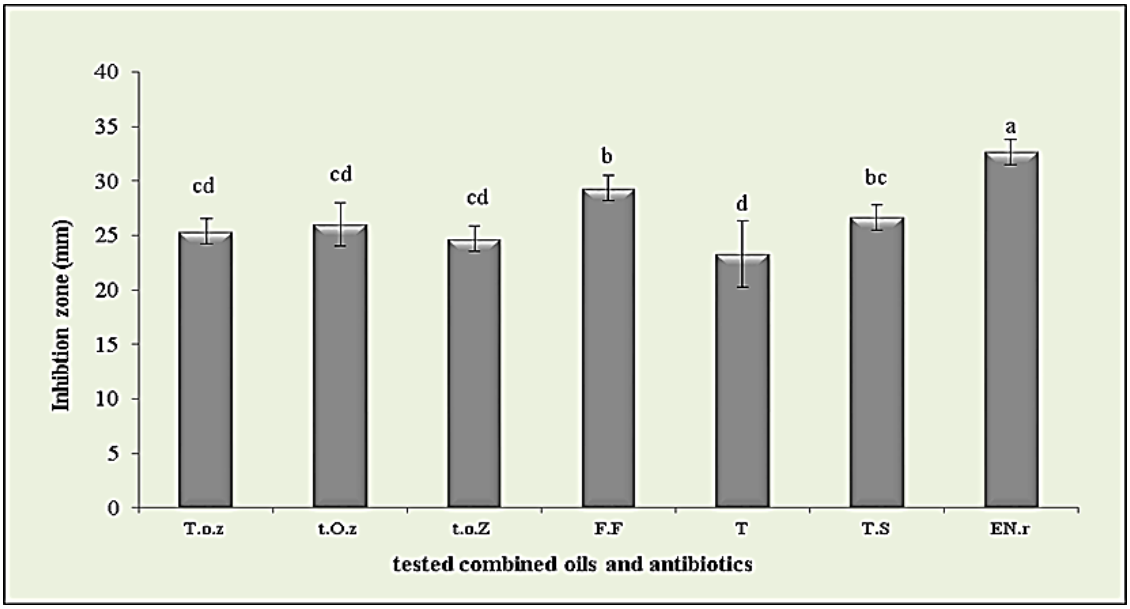

Figure 1. Disc diffusion results against *Aeromonas hydrophila* (disc diameter = 6 mm- included) (FF = Florfenicol, T = Oxytetracycline, T.S = Trimethoprim sulfamethoxazole and EN.r= Enrofloxacin).

essential oil, 17 compounds were identified accounting for 99.06% of the total oil. Carvacrol (or Isothymol) (57.86%), Thymol (13.54%), Trans-Caryophyllene (11.52%) and Cymene (6.78%) were major compounds (Table 1) and the minimum quantity was 0.08% for α-Thujene. For *Z. clinopodioides*, α-Pinene (22.6%), Carvacrol (21.1%), 1.8-Cineole (9.1%), Limonene (8.3%), Cymol (8.2%) and Thymol (7.0%) were major compounds out of 27 (Table 1). A remarkable richness in monoterpenes compared to sesquiterpenes was observed in the studied essential oils.

Antibacterial effects: All the tested CEOs exhibited an antibacterial effect by disc-diffusion test (Fig. 1). Among them, t.O.z (with 50% *O. majorana*) presented the best inhibition zone (26 mm), followed by T.o.z (25.33 mm) and t.o.Z (24.66 mm). But no significant differences were found between them (P<0.05). Indeed, when the effects of COEs were compared to the synthetic antibiotics (i.e. four antibiotics), Oxytetracycline had the lowest efficacy, whereas Enrofloxacin (inhibition zone= 32.66 mm) showed priority compared to other CEOs (P<0.05).

The results of MIC and MBC are shown in Table 2. The T.o.z and t.o.Z exhibited the same inhibitory activity (MIC=0.8 µL /mL), while that of the t.O.z was the lowest (MIC=0.4 µL /mL). Regarding the bactericidal effect, all CEOs showed similar results (MBC=3.2 µL /mL).

Bacterial growth curves: The growth curves of *A. hydrophila* under T.o.z treatment were significantly uneven in both treated with CEOs and control group at all tested temperatures (Fig. 2 A, B, C). Significant differences were found in the exponential phase of growth curves, but in the Lag duration i.e. Lag phase, *A. hydrophila* growth in either essential oils TSB (Tryptic Soy Agar) or blank TSB

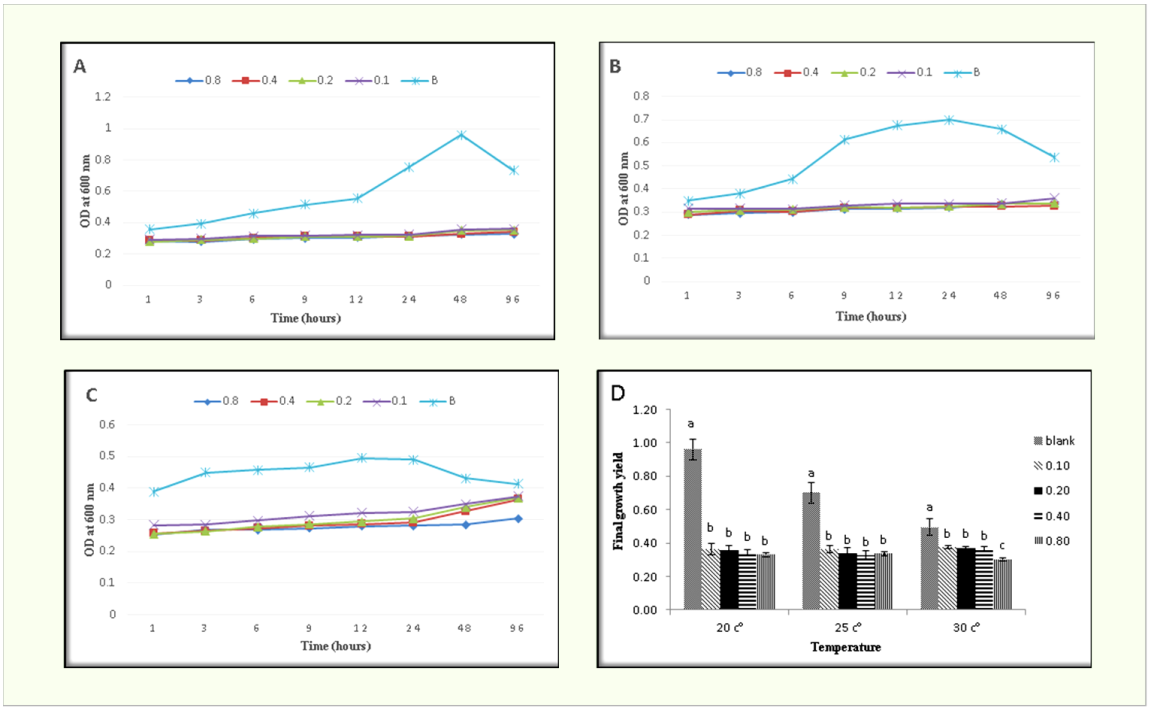

Figure 2. Bacterial growth curves (based on OD at 600 nm) under various concentrations of T.o.z (50% *T. vulgaris*) essential oil at temperatures of 20 (A), 25 (B) and 30°C (C) for *Aeromonas hydrophila*. Growth yields in the previous conditions (D). (Approved concentrations of combined essential oil were less than MBC and ranged between 0.1 to 0.8 µL /mL and B (blank) = no essential oil; values are means (n=5). A, b, bc, and c = standard deviation).

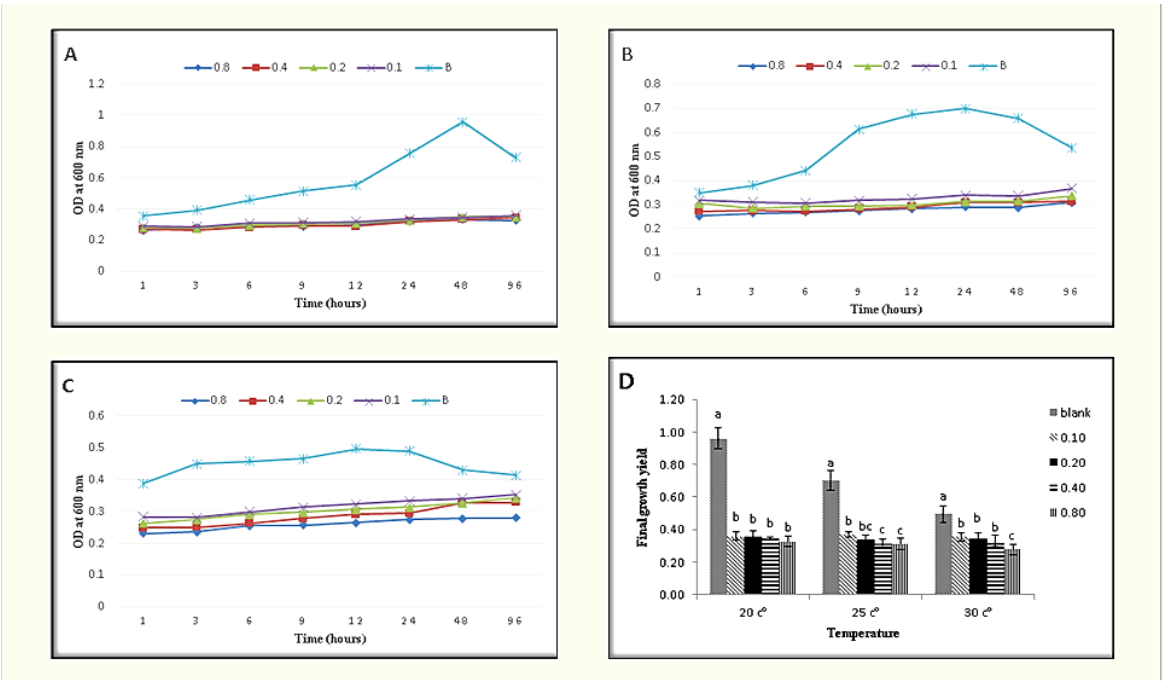

Figure 3. Bacterial growth curves (based on OD at 600 nm) under various concentrations of t.O.z (50% *O. majorana*) essential oil at temperatures of 20 (A), 25 (B) and 30°C (C) for *Aeromonas hydrophila*. Growth yields in the previous conditions (D). (Approved concentrations of combined essential oil were less than MBC and ranged between 0.1 to 0.8 µL /mL and B (blank) = no essential oil; values are means (n=5). A, b, bc, and c = standard deviation).

(B) did not affect by temperature, suggesting that different temperatures neither stimulated nor suppressed the duration of the Lag phase. In addition, the differences in the growth curves were apparent

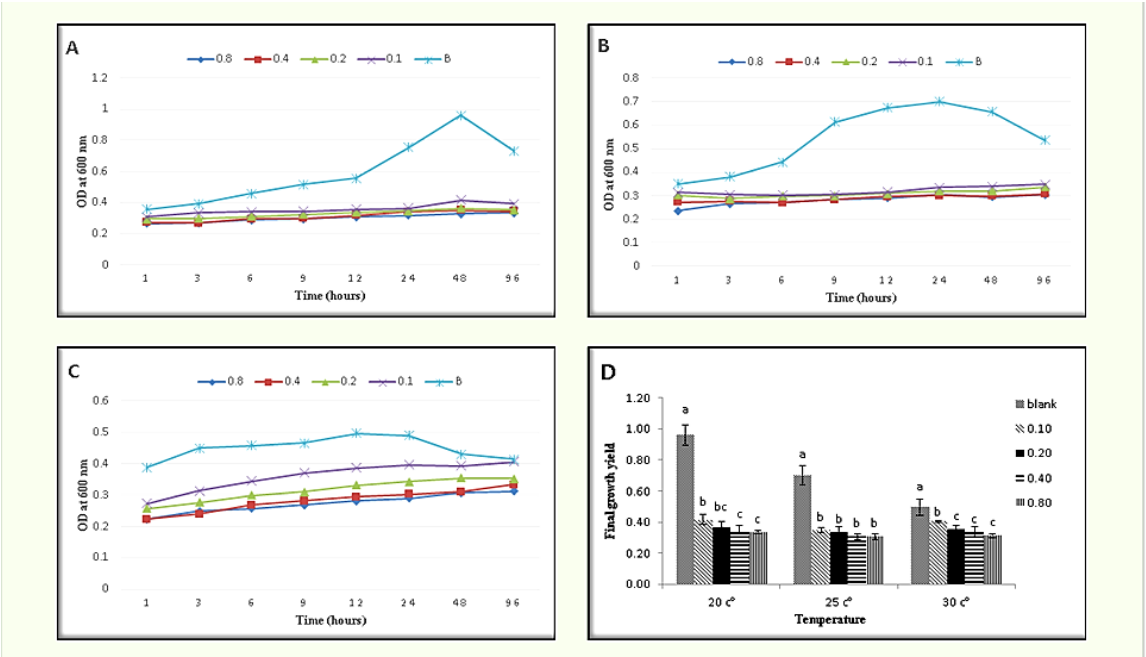

Figure 4. Bacterial growth curves (based on OD at 600 nm) under different concentrations of t.o.Z (50% *Z. clinopodioides*) essential oil at temperatures of 20 (A), 25 (B) and 30°C (C) for *Aeromonas hydrophila*. Growth yields in the previous conditions (D). (Approved concentrations of combined essential oil were less than MBC and ranged between 0.1 to 0.8 µL /mL and B (blank) = no essential oil; values are means (n=5). A, b, bc, and c = standard deviation).

even in the lowest concentrations of T.o.z , confirmed by the highest points of the growth curves and further bacterial growth yields i.e. a significant differences between T.o.z treatments and blank one (B=TSB with no essential oil) were observed (Fig. 2D). OD-value reached 0.960 nm at 48 h as the final growth yield in blank group i.e. without essential oil at 20°C, whereas it was 0.363 nm at 96 h in the lowest T.o.z concentration (=0.1 µL /mL). In the bacterial growth curves at 25°C with the previous conditions, the final growth yield was 0.700 nm for blank at 24 h reaching 0.360 nm in 0.1 µL /mL of T.o.z at 96 h. In the final growth yield at 30°C, OD was 0.496 nm at 12 h for blank, reaching 0.375 nm at 96 h for the lowest concentration of CEO.

The bacterial growth curves for *A. hydrophila* in t.O.z treatments i.e. 50% *O. majorana*, essential oil and control were similar to those of T.o.z (Fig. 3A, B, C) where, OD reached 0.960 nm at 48 h as the final growth yield in the blank situation at 20°C. It was 0.360 nm at 96 h in the lowest t.O.z concentration (=0.1 µL /mL). In the bacterial growth curves at 25°C with the previous conditions, the final growth yield measured 0.700 nm for blank at 24 h reaching 0.368 nm in 0.1 µL /mL of t.O.z at 96 h. Regarding the final growth yield at 30°C, OD was 0.496 nm at 12 h for blank, while it reached 0.352 nm at 96 h for the lowest concentration of CEO. At all blank (B) situations, a significant differences (P<0.05) were found comparing to various concentrations of essential oil (Fig. 3D).

The t.o.Z showed the weakest effect at 30°C (Fig. 4C). OD-value at 30°C reached 0.496 nm at 12 h as the final growth yield for *A. hydrophila* with no essential oil (blank), whereas it was 0.420 nm at 96 h in the lowest t.o.Z concentration (=0.1 µL /mL). In contrast, these two values were almost similar, but significantly different (P<0.05) (Fig. 4D). The bacterial growth curves at 20 and 25°C were similar to those of T.o.z and t.O.z (Fig. 4A, B).

Discussions

Phytochemicals is considered as secondary metabolites of low-molecular weight occurring naturally in plants. These biologically active molecules have a main role in the interaction between the plant and its environment, i.e. they serve as a defense against insects, fungi, and other

microorganisms, as growth regulators, pigments, and flavors (Leitzmann, 2016). With the discovering of the medical properties of the herbal extracts, most recent research focused to investigate their effects on pathogenic organisms such as bacteria and fungi.

Based on the results, the major components of *T. vulgaris* were thymol, limonene, cymene and α-terpinolene. This finding was in agreement with those reported by Al-Asmari et al. (2017), Cosentino et al. (1999), Divband et al. (2017) and Yılmaz et al. (2010). However in other reports, the Borneol and α-terpineol have been found as major compounds of *T. vulgaris* (Radaelli et al., 2016). This difference shows that its compound concentrations affected by several factors, including geographic areas, climatic conditions, season of the plant collection, species, growth stages, origin of herb, drying conditions and distillation conditions (Nhu-Trang et al., 2006). In addition, every component in essential oil composition has different effects. For instance, the antioxidative effect of thyme is based on polyphenolic compounds as flavonoids (Luteolin), while Thymol and Carvacrol have high antimicrobial activity (Justesen and Knuthsen, 2001).

In the *O. majorana* essential oil, the most abundant compounds were Carvacrol, Thymol, Trans-caryophyllene and Cymene. This result is contrast to previous reports (Tabanca et al., 2004; Mossa and Nawwar, 2011; Dantas et al., 2016) that Terpinene, Terpineol and Sabinene were major compounds. Although they were conducted in different geographic regions, but their data were similar regarding the reported components, suggesting that differences in geographic areas cannot always affect herbal oil composition. In other study, the Thymol and/or Carvacrol were found to be the predominant compounds as same as of the present study (Banchio et al., 2008).

The α-pinene, Carvacrol, 1.8-cineole, Limonene, Cymol and Thymol were respectively exhibited as the major constituents in the *Z. clinopodioides* essential oil and this is consistent with the rsults of Shahbazi (2017), who investigated the composition of *Z. clinopodioides* in the three geographical region of Iran showing that thymol was the highest compound in some areas, while Carvacrol in others. However in previous studies, the Pulegone reported as the most abundant component (Ding et al., 2014; Kheirkhah et al., 2015). The studied essential oils have a remarkable richness in polyphenols (such as phenolic acids and flavonoids) and monoterpenes (for instance Menthol, Carvone and Limonene).

The therapeutic agents as antibiotics and various chemicals have been used against *A. hydrophila* that have been led to bacterial resistance for antibiotics. Application of the plant products such as *Thymus* and *Origanum* sp. essential oil, as therapeutic agents has been well-known (Aligiannis et al., 2001; Baranauskienė et al., 2003; Baydar et al., 2004). The results showed that t.O.z exhibited the best inhibition zone (26 mm) but had no significant differences with other CEOs. Its superiority can be explained due to having a higher concentration of the Carvacrol and Thymol. The hydroxyl group of Thymol and Carvacrol and the presence of a system of delocalized electrons in their chemical structure play a major role in their antibacterial effects (Nazzaro et al., 2013). Thymol interacts with cell membrane affecting its permeability leading to the loss of membrane potential, cellular uptake of ethidium bromide and leakage of potassium ions, ATP and carboxyfluorescein. The antimicrobial effect of the Carvacrol is expected to be similar to the thymol, causing functional and structural damages to cell membrane (Ouedrhiri et al., 2016).

According to MIC and MBC results, t.O.z has the superiority to other COEs. This can be explained by more Carvacrol content in t.O.z, which has been previously reported having high inhibitory and bactericidal effects. In addition, the Cymene in *O. majoran* potentiates the antibacterial effect of the Carvacrol (Ultee et al., 2002). This shows that combination of the herbal essential oils leads to synergistic effect between their (not at all situations), saving essential oil consumption.

Recently, there is a high interest in bacterial growth laws (Bren et al., 2013). Bacteria respond to

environmental changes by reprogramming their metabolism (Baev et al., 2006). The presence of the herbal oils in growth media and alternation in temperature can be considered as environmental changes. The bacterial growth curve consists of three phases, including Lag (initial), Log (exponential) and stationary (deceleration) (Bren et al., 2013). Bren et al. (2013) showed that deceleration phase exist and the main reason of its presence is nutrient limitation in growth media. This is in accordance with our results especially for bacterial growth curves in blanks (with no essential oils). In particular, deceleration phase was occurring in different cases after 24 or 48 h. Furthermore, in the present study significant differences was found between *A. hydrophila* growth curves with the presence of combined herbal oils and its absence particularly, in exponential phase at all conditions. Based on the results, we can point out that in most cases (under CEOs treatment), *A. hydrophila* exhibits a significant growth comparing to blanks. Moreover, increasing the growth curves in some situation e.g. in T.o.z at 30°C is occurred after 24-48 h. Hence, it is suggested that the mixing the herbal oils with growth media was delaying exponential phase starting in the bacterial growth curves.

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