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Efficacy of herbal extracts to control multi-antibiotics resistant (MAR) *Aeromonas veronii* isolated from motile *Aeromonas* septicemia (MAS)-Exhibiting Nile tilapia (*Oreochromis niloticus*)

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Abstract Motile *Aeromonas* septicemia (MAS) is a disease causing a devastating loss in fish farming including Nile tilapia (*Oreochromis niloticus*). The disease is caused by pathogenic bacteria *Aeromonas* spp. Our previous study has revealed that *A. verronii* is one of commonly isolated species from the MAS–expressing Nile tilapia cultured in Southern Thailand. Among the collected bacteria, one isolate exhibiting multi-antibiotic resistance (MAR) against tetracycline and oxyteracycline was selected as a model for testing the biological control ability of herbal extracts. The herbal extracts obtained from 6 herbal plants; *Caesalpinia sappan* (Cs), *Allium sativum* (As), *Illicium verum* (Iv), *Alpinia galanga* (Ag), *Piper longum* (Pl), and *Foeniculum vulgare* (Fv), extracted using 3 different solvents; 95% ethanol (E95), water (W) and soybean oil (O) were first screened for their inhibitory potential by disc diffusion method compared with tetracycline (30 μg). The clear zones (7–18 mm) were detected in Cs-E95, Cs-W, As-E95, Iv-E95, IV-W, and Ag-E95 while the clear zone in tetracycline was about 13 mm. All 6 herbal extracts showing clear zone were further evaluated for their minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against the MAR–*A. veronii*. The greatest MIC of 0.469 mg/ml was recorded in Cs-E95 and Cs-W and MBC of 0.938 mg/ml in Cs-W. Moreover, the inhibitory effect of Cs-E95, at the concentration of MIC and MBC, on the growth of MAR–*A. veronii* cultured under the water temperature observed in the fish farm was also demonstrated. This finding revealed the potential of herbal extracts as a favorably health and environmental friendly approach in modern aquaculture to control the bacterial pathogens instead of using antibiotics.

Keywords: motile *Aeromonas* septicemia (MAS), *Oreochromis niloticus*, multi-antibiotics resistance (MAR), herbal extract, *Aeromonas veronii*

Introduction

The aquaculture and fish farming has been growing very fast because of the increasing of world fish consumption. Nile tilapia, *Oreochromis niloticus*, is one of the important fish for freshwater aquaculture especially in Thailand. The

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trend of tilapia culture nowadays is an intensive system in order to obtain the highest production value. As a result, the culturing environments become factors those enhance the susceptibility of fish while strengthen the pathogens (Naylor *et al*., 2000; Cabello *et al*., 2006). Therefore, the improvment of tilapia culture to gain the great production and disease resistance against pathogens is a major challenge facing fish farmers. The important disease affecting a devastative loss of tilapia culture is motile *Aeromonas* septicemia (MAS) caused by bacteria in the genus *Aeromonas*. *A. hydrophila*, *A. caviae*, *A. salmonicida* and *A. veronii* have been considered to cause tail and fin rot, hemorrhagic septicemia and ulcerative syndrome in various freshwater species (Abd-El-Rhman, 2009; Scarano *et al*., 2018).

Antibiotics have been used as powerful tools to overcome the bacterial diseases in aquaculture. In addition, antibiotics are regularly applied as additives in fish food or as prophylactics and therapeutics (prevent disease and treat sick animals, respectively) or growth promoters (Cabello *et al*., 2006; Rico *et al*., 2013). Long-term and excessive use of antibiotics has resulted in numerous side-effects regarding the environment and human health. One of the critical problems that has to be concerned is the development of antibiotics resistant bacteria. There are several alternative approaches in aquaculture to reduce the use of antibiotics, for example, enhance the strength or the immunity of the animals and discover the natural products or substances as well as probiotics which exhibit promising potential to kill or control the pathogens (Zahran *et al*., 2014). In addition, the organic and environmentally friendly food becomes importantly concerned in aquaculture products because of the consumers' favor. The extracts naturally obtained from plants or herbal plants have thus been increasingly interested in this regard.

Plants comprise a wide range of chemical substances including diverse phytochemicals that can be used for traditional medicine in regard to treat either chronic or infectious diseases (Duraipandiyan *et al*., 2006). The extracts originated from plants have been studied for their diverse potentials since the sources are considerably safe and less adverse effects compared to antibiotics or chemicals (Reverter *et al*., 2014). The first ability of plant extract is being used as appetite and growth promoters, for example, garlic administration to Nile tilapia showed increase of food intake, final weight and specific growth rate (Shalaby *et al*., 2006). Moreover, there have been demonstrated the immunostimulatory activity of plant extracts to enhance the animal immunity especially throurh the non-specific or innate immune system (Düğenci *et al*., 2003; Oskoii *et al*., 2013). The apparent benefit of plant extracts applied in aquaculture is antibacterial substances. Their antibacterial activities against several pathogenic bacteria have been shown *in vitro* and *in vivo*. Castro *et al*.

(2008) have reported that methanolic extracts of Brazilian plants exhibit the *in vitro* antibacterial activity agaist *Streptococcus agalactiae*, *Flavobacterium columnare* and *A. hydrophila*, the important fish pathogenic bacteria. The *in vivo* antibacterial activity of plant extracts, eggplant (*Solanum trilobatum*) and Chinese cedar (*Toona sinensis*), has also been shown in tilapia against *A. hydrophila* since the mortality is lower in the trated fish compared to control untreated fish (Divyagnaneswari *et al*., 2007; Wu *et al*., 2010).

Based on our previous study, the antibiotic resistant bacteria belonging to the genus *Aeromonas* have been isolated from the MAS–exhibiting Nile tilapia cultured in Southern Thailand. Among the collected *Aeromonas* spp., the profile and degree of antibiotics resistant against tetracyclines was detected. One isolate was selected to further classification since it was charaterized as a multi-antibiotic resistant (MAR) strain. This present study aimed to investigate the *in vitro* antibacterial potential of herbal plant extracts to containment the growth of pathogenic bacterium exhibiting multi-antibiotic resistance. The obtained results elucidate the appropriate herbal extract which is worthy of antibacterial natural product development applied in aquaculture.

Materials and methods

Pathogenic bacterium, molecular characterization and antibiotic resistance profile

An isolate of bacterium showing Gram-negative and short rod-shaped showing antibiotic resistance was classified based on molecular aspect. This isolate was cultured in Tryptic Soy Broth (TSB, Difco) for 18 h then the cell was separated for DNA extraction using Presto™ Mini gDNA Bacteria Kit (Geneaid). PCR was conducted using a primer set, 20F: 5′-AGAGTTTGATCATGGCTCAG -3′ and 1500R: 5′-CGGTTACCTTGTTACGACTT -3′ (Weisburg *et al*., 1991) to verify 16S rDNA. Cycling condition, denaturation at 94 ℃ for 5 min, followed by 35 cycles of [95 ℃/1 min-55 ℃/1 min-72 ℃/1 min] and a 10-min extension at 72 ℃ was performed in a T100™ Thermal Cycler (Bio-Rad). The amplified product was purified, ligated into the pGEM®-T Easy Vector (Promega) and transformed into *Escherichia coli* Top10 cells. Purified plasmids were sequenced and the obtained result was used for genus and species identification according to multiple sequence alignment (ClustalW) (Thompson *et al*., 1994) and phylogenetic tree construction using MEGA X.

The bacterium was checked for its antibiotic resistance profile against the drugs in tetracycline group including oxytetracycline (OT), tigecycline (TGC),

doxytetracycline (DO), tetracycline (TE) and minocycline (MN), through disc diffusion method (Abd-El-Rhman, 2009).

The tested *Aeromonas* sp. was adjusted with 0.85% (w/v) NaCl to the desired concentration equal to McFarland Standard No. 0.5 before spreading on Mueller-Hinton (MH) agar, then the disc conatianing drug (6 mm in diameter) was placed under aseptic technique. The inhibition zones measured after 24 h incubation were present in mellimeters and the antibiotic susceptibility was considered in accordance with the interpretive criteria standard of the manufacturer, Oxoid Ltd, which referred to CLSI document M100-S23 (M02-A11) and Clinical and Laboratory Standards Institute (CLSI, 2012).

Preparation of herbal extracts

Six herbal plants; *Caesalpinia sappan* (Cs), *Allium sativum* (As), *Illicium verum* (Iv), *Alpinia galanga* (Ag), *Piper longum* (Pl), and *Foeniculum vulgare* (Fv), were minced and immersed in 95% (v/v) ethanol (E95), water (W) or soybeen oil (O) as extraction solvents with the ratio of 1:10 for 72 h. Therefore, there were 18 herbal extracts (Cs-E95, Cs-W, Cs-O, As-E95, As-W, As-O, Iv-E95, Iv-W, Iv-O, Ag-E95, Ag-W, Ag-O, Pl-E95, Pl-W, Pl-O, Fv-E95, Fv-W and Fv-O) in this study. The herbal debris were separated and the ethanolic and water extracts were evaporated and lyophilyzed, respectively, while the oil extracts were immediately collected after the debris separation. All herbal extracts were kept at –20^{o}C until used for further study.

Screening for the antibacterial activity of the herbal extracts

The disc diffusion method following Abd-El-Rhman (2009) was employed to determine the antibacterial potential of the obtained herbal extracts. The MAR–*Aeromonas* sp. was prepared as mentioned above. All herbal extracts, except those extracted by oil, were prepared at a concentration of 100 mg/ml, using 50% (v/v) ethanol as the solvent, and then filtered through a 0.45 μm membrane filter (Millipore). Filter-paper-discs were impregnated with 30 μl of each filtered herbal extract and either 50% (v/v) ethanol or oil was used as control. In addition, the disc of TE (30 μg) was used as a positive control.

Only herbal extracts showing the clear zone as did the positive TE were considerably potent antibacterial substances selected for further determination more insight of antibacterial activity.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

MIC of each potent herbal extract was determined through broth microdilution method following Baron *et al*. (2017) with silght modification. MIC determination was performed in the 96-well microplate; each well contained solution of herbal extract which was two-fold diluted to the final concentration in a range of 0.015 to 30 mg/mL and 2.5 x 10^6 CFU/ml of MAR–*Aeromonas* sp. The MIC of the antibitic TE was also evaluated in the range of 0.015 to 30 μg/ml. Three wells conteined only bactetial suspenton and three of sterile herbal extract (no bacteria) were designed as positive and negative control, respectively. After incubation at room temperature for 24 h, the optical density (OD) at 600 nm was measured. The OD_{600} of the tested wells was subtracted with the negative control, at the same dilution, prior to being evaluated for the MIC. The MIC was indexed at the least-concentration-well showing the OD_{600} remarkably beneath the positive control.

Viable cell enumeration was conducted to establish the MBC value (Starliper *et al*., 2015). The bacteria in the wells of MIC and lower-dilution wells, which exhibit the same phenomenon, were subcultured on Tryptic Soy Agar (TSA, Difco) and incubated for 24 h. The lowest concentration that showed no bacteria growing on the plate was specified as MBC.

Growth of bacteria cultured at MIC and MBC under the water temperature observed in the fish farm

The MAR–*Aeromonas* sp. was cultured in the TSB containing the high potential of antibiotic herbal extract at its MIC and MBC and no herbal extract as control. Prior to testing, the bacteria was subcultured twice to a new TSB then transferred to a new culture medium containing TSB and 0.85% (w/v) NaCl (1:1) and herbal extract at the desired concentration as described above. The culturing temperature was based on the water temperature (24–30^{o}C) typically observed in the fish farm. The growth curve and specific growth rate was real-time monitored for 24 h using RTS-1C Personal bioreactor (Biosan).

Results

The molecular characterization based on the partial sequence of 16S rDNA sequence (approximately 1,500 bp) indicated that the randomly selected Gram-negative and short rod-shaped bacterium exhibiting antibiotic resistance was *A. veronii*. The obtained sequence was perfectly matched with 16S rDNA of other *A.veronii* isolates deposited in the GenBank (Figure 1). Moreover, the phylogenetic tree constructed using the partial sequences of 16S rDNA of

Aeromonas spp., *Enterobacteriaceae* and out group of *Bacillus* spp. is shown in Figure 2.

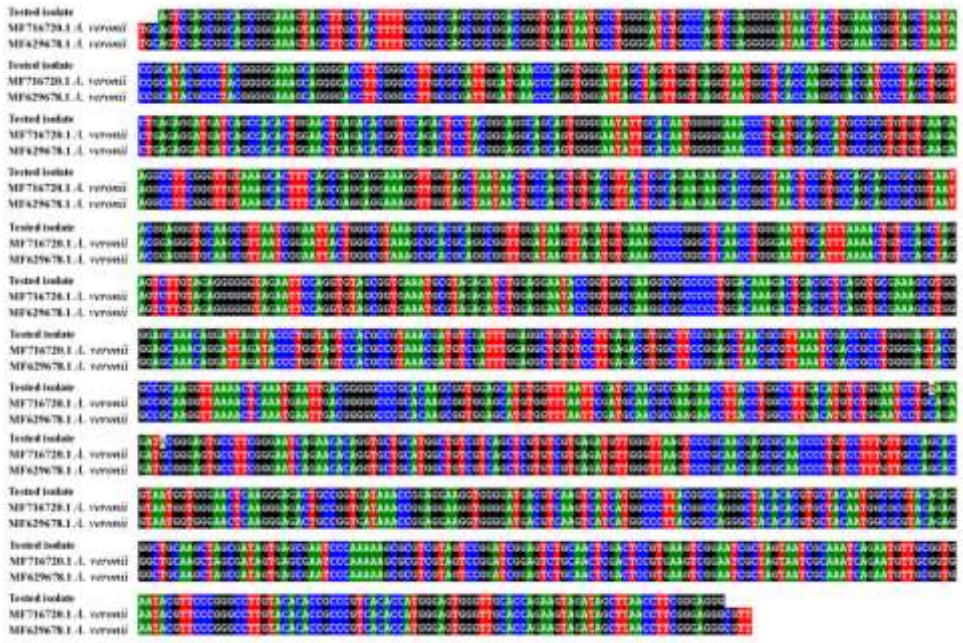

Figure 1. Nucleotide sequence alignment of the partial sequences of 16S rDNA from the *Aeromonas* sp. identified in this study and other *A. veronii* submitted in the GenBank

Table 1. Antimicrobial susceptibility results of the tested *A. veronii*

Sample	Inhibition zone diameter (mm)[1]					Antibiotic resistance profile
	OT (30 µg)	TGC (15 µg)	DO (30 µg)	TE (30 µg)	MN (30 µg)	
A. veronii	10.0 (R)	22.1 (S)	19.1 (S)	11.0 (R)	23.9 (S)	OT/TE

Antimicrobial susceptibility; OT, Oxytetracycline; TGC, Tigecycline; DO, Doxytetracycline; TE, Tetracycline; MN, Minocycline.

[1/] Determined by disc diffusion method. The interpretive criteria provided by Oxoid Ltd referred to the Clinical and Laboratory Standards Institue (CLSI). Inhibition zone in mm of each antibiotic; OT, Resistant (R) ≤ 17, Intermediate (I) 18–21, Susceptible (S) ≥ 22; TGC, R ≤ 14, I 15–18, S ≥ 19; DO, R ≤ 10, I 11–13, S ≥ 14; TE, R ≤ 11, I 12–14, S ≥ 15; MN, R ≤ 14, I 14–15, S ≥ 16.

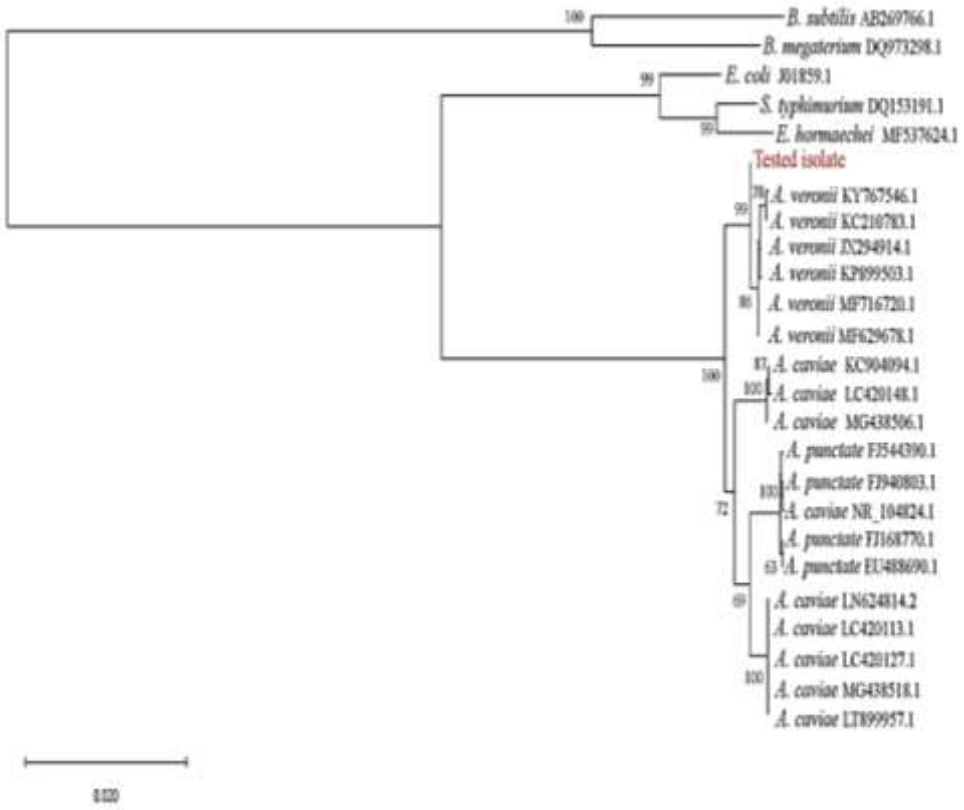

Figure 2. Phylogenentic analysis based on nucleotide sequences of 16S rDNA from the tested isolate *Aeromonas* identified in this study, other *Aeromonas* spp. and *Enterobacteriaceae* while *Bacillus* spp. were used as outgroup. The phylogenetic tree was constructed via Neighbor-joining with 1000 bootstraps (MEGA X) Bootstrap values greater than 50% are shown at the branch point

The efficiency of the ethanolic, water and oil extracts of 6 herbal plants to control the MAR–*A. veronii* was evaluated by disc diffusion method using TE (30 μg) as the positive control. The variation of antibacterial activity of the herbal extracts was observed (Table 2). Inhibition zones was recorded around the discs impregnated with Cs-E95 (~18 mm), Cs-W (~14 mm), As-E95 (~17 mm), Iv-E95 (~7 mm), Iv-W (~8 mm), Ag-E95 (~11 mm) while TE exhibited the clear zone in diameter of approximately 13 mm.

Table 2. Inhibition zone diameter (mm) of the MAR–*A. veronii* caused by various herbal extracts

Herbal plant/ Antibiotic	Extraction solvent	Name of herbal extract	Inhibition zone diameter (mm)[1]
Caesalpinia sappan (Cs)	95% Ethanol (E95)	Cs-E95	18.65±0.60
	Water (W)	Cs-W	14.63±0.28
	Soybean oil (O)	Cs-O	ND
Allium sativum (As)	95% Ethanol (E95)	As-E95	16.75±0.50
	Water (W)	As-W	ND
	Soybean oil (O)	As-O	ND
Illicium verum (Iv)	95% Ethanol (E95)	Iv-E95	7.17±0.13
	Water (W)	Iv-W	7.83±0.33
	Soybean oil (O)	Iv-O	ND
Alpinia galanga (Ag)	95% Ethanol (E95)	Ag-E95	10.98±0.28
	Water (W)	Ag-W	ND
	Soybean oil (O)	Ag-O	ND
Piper longum (Pl)	95% Ethanol (E95)	Pl-E95	ND
	Water (W)	Pl-W	ND
	Soybean oil (O)	Pl-O	ND
Foeniculum vulgare (Fv)	95% Ethanol (E95)	Fv-E95	ND
	Water (W)	Fv-W	ND
	Soybean oil (O)	Fv-O	ND
Tetracycline (TE, 30 μg)			13.23±0.15

Abbreviation: ND, not determined.
[1/] Mean±SD of inhibition zone diameters (n=6) determined by disc diffusion method.

Six potent herbal extracts, which exhibited the inhibition clear zone, including Cs-E95, Cs-W, As-E95, Iv-E95, Iv-W, and Ag-E95, were subjected to determine MIC and MBC values to get more insight in the antibacterial activity against the MAR–*A. veronii*. Broth microdilution was employed and the results are present in Table 3. The MIC values were varied among the tested herbal extracts. The lowest MIC (0.469 mg/ml) was recorded in the Cs-E95 and Cs-W followed by As-E95 and Ag-E95 (1.875 mg/ml) and Iv-E95 and Iv-W (7.500 mg/ml). The MBC values were corresponding with those of the MIC.

Interestingly, in case of Cs-W and Cs-E95 which showed the aqual MIC value, Cs-W gave lower MBC (0.938 mg/ml) than that of Cs-E95 (1.875 mg/ml).

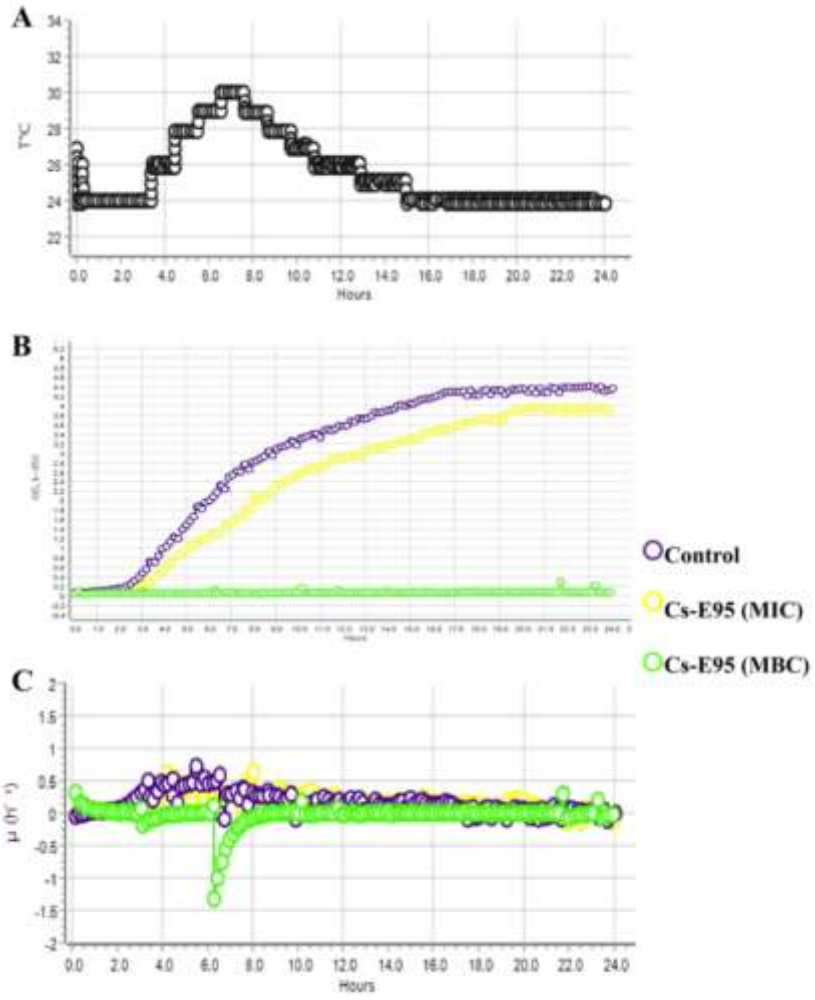

Figure 3. Effect of the herbal extract (Cs-E95) on the growth of MAR–*A. veronii* cultured under diurnal water temperature. (A) Temperature profile of the diurnal water temperature mimicking that in the fish farm, (B) Growth curve and (C) Specific growth rate of the MAR–*A. veronii* cultured in the media contain no herbal extract as a control, Cs-E-95 at MIC and Cs-E95 at MBC

Table 3. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the potent herbal extracts against the MAR–*A. veronii*

Name of the potent herbal extract/Antibiotic	MIC[1]	MBC[1]
Cs-E95	0.469 mg/ml	1.875 mg/ml
Cs-W	0.469 mg/ml	0.938 mg/ml
As-E95	1.875 mg/ml	7.50 mg/ml
Iv-E95	7.50 mg/ml	15.00 mg/ml
Iv-W	7.50 mg/ml	15.00 mg/ml
Ag-E95	1.875 mg/ml	7.50 mg/ml
TE	1.875 μg/ml	15.00 μg/ml

[1/] Determined by broth microdilution.

After the MIC and MBC values were obtained, the Cs-E95 was selected for studying the effect of the herbal extract on the growth behavior of the MAR–*A. veronii* cultured under the diurnal water temperature mimicking that in the fish farm. The water temperature profile vaired between 24^{o}C and 30^{o}C is shown in Figure 3A. As expected, the growth curve was lower in the culture media containing Cs-E95 at MIC compared to the control (no herbal extract) while it seemed to be stable (no change) in the treatment of Cs-E95 at MBC (Figure 3B). Among the specific growth rate, the distinct profile was observed in the Cs-E95 at MBC whereas control and Cs-E95 at MIC showed the similar pattern (Figure 3C).

Discussion

MAS caused by *Aeromonas* spp. of family Aeromonadaceae is considered as the important disease in aquaculture (Wahli *et al*., 2005). Although *A. hydrophila* has been reported as a species generally isolated from the diseased freshwater fish and environemt (Daskalov, 2006; Ye *et al*., 2013; Vidhya Hindu *et al*., 2018), the bacterium isolated from the MAS–exhibiting Nile tilapia in Southern Thailand in this study was characterized as *A. veronii* based on 16S rDNA sequence and phylogenetic tree construction. This molecular technique has been proven as a reliable tool to identify genus and species of bacteria besides the biochemical characterization (Janda and Abbott, 2007; Singh *et al*., 2012).

For decades, antibiotics have been used to overcome the pathogenic bacteria *Aeromonas* spp. However, it has to be concerned because of the risen awareness of the adverse effects. There has been increasing in the literacy regarding the antibiotic resistant bacteria including *Aeromonas* spp. against various types of antibiotics (Janda and Abbott, 2010). This research therefore studied the antibiotic resistance profile of the collected *A. veronii*. Five antibiotics in tetracycline group were tested since use of tetracyclines is allowed in aquaculture in Thailand. The result indicated that our interested *A. veronii* belongs to MAR strain with the antibiotic resitance profile of OT/TE according to the inhibition zones.

Because of the appearance of antibiotic resistant bacteria, the ability of antibiotics becomes suspicious. The plant extracts are thus an alternative appoarch to potentially control the bacterial pathogens (Reverter *et al*., 2014). Several plants have been extracted for their bioactive compounds using different solvents and shown the antibacterial activity (Sasidharan *et al*., 2011). The solvents commonly used to extract the herbal or medicinal plants are water, oil, ethanol, and methanol, however, the latest is higher toxic and its residue may be problematic. The herbal extracts used in this present works were therefore extracted by water, oil and ethanol. The obtained results indicated that extracts using ethanol gave higher antibacterial activity than those of water and oil since the 4 of 6 herbal ethanolic extracts showed inhibition zone. Two herbal extracts obtained from water extraction could show antibacterial activity while only one from oil extract could be observed for the antibacterial potential. This maybe due to the antibacterial active compounds were differently distilled by the diverse solvents. It is noticeably that water extracts contain a group of polar compounds, including carbohydrate and proteins, rather than secondary metabolites such as phenolic substances (Saifudin *et al*., 2016).

The plants used in this study have also been demonstrated for their antimicrobial potential. However, this present study was the first report that demonstrating the antibacterial activity of the plant extracts against the MAR bacterium strain. *C. sappan* extracted by ethanol, water and petroleum showed inhibition zone when testing with *Pseudomonas aeruginosa* and *E. coli* (Srinivasan *et al*., 2012). The extract of *I. vercum* characterized as anethole exhibited the antimicrobial activity against bacteria, yeast and fungi (De *et al*., 2002). The extract named allicin from garlic (*Allium sativum*, Allimed®) showed the potential to control the growth of *A. hydrophila* (Nya *et al*., 2010). Correspondingly, the extracts from these 3 plants also showed the effective against MAR–*A. veronii*. In case of *A. galanga*, the previous study by Chan *et al*. (2011) testing with *Staphylococcus aureus*, *Micrococcus luteus*, and *Bacillus cereus* was parallel to our result that its extract exhibited poor

antimicrobial activity. In addition, diverse plant extracts have been demonstrated differently in MIC and MBC values. Linga Prabu *et al*. (2018) have demonstrated the MIC and MBC of 11 different methanolic plant extracts against *A. hydrophila*; for example, *Phyllanthus acidus* (12.5 and 25 μg/ml), *Syzygium cumini* (3.12 and 6.25 μg/ml), *Acalypha indica* and *Solanum nigrum* (25 and 50 μg/ml), *Sesbania grandiflora* and *Cassia angustifolia* (50 and 100 μg/ml). Hammer *et al*. (1999) have reported that the essential oil of oregano (*Origanum vulgare*), lemongrass (*Cymbopogon citratus*) and thyme (*Thymus vulgaris*) yielded MIC of 0.12% against *A. sobria*. However, there have been mentioned that MIC and MBC values are varied depends on the analysis methods (Arthington-Skaggs *et al*., 2002). Furthermore, the results of antimicrobial acitivity reported by several researches have been considerably difficult to compare directly, usually due to the distinct of number or concentration of palnt samples tested, diverse bacterial strains as well as the origin of tested microbials. The lowest MIC and MBC in this study was present in *C. sappan* extracted with 95% ethanol and water (CS-E95 and Cs-W) which showing strong antibacterial efficiency. This might be due to the constituents in the extracts. Srinivasan *et al*. (2012) have reported the phytochemical compoments in exracts of *C. sappan* were composed of high contents of steroids, tannin, phenol, fixed oil and fats, saponins and flavonoid in the ethanolic extract while low tannin and moderate phenol in aqueos extract.

To further confirm the efficacy of the herbal extract on controlling the growth of bacteria, the OD determined in the broth of bacteria culture was measured. Differently, this study is the first evidence reporting the effect of herbal extract on the real-time growth of bacteria while others used the method of sampling the cultured media to measure the OD (Krajčová *et al*., 2008; Tu *et al*., 2018). Moreover, to the best of our knowledge this is the first time demonstrating the effect of the herbal extract on the bacterial growth by means of growth curve and specific growth rate under the diurnal temperature change. We aimed to illustrate the patterns of bacterial growth influenced by the diurnal temperature and herbal extract, Cs-E95, at the concentration of MIC an MBC. The bacterial inhibition potential was observed; lag phase was prolonged, delayed of active growth and the total bacteria was lower in the mediun containing Cs-E95 at MIC, similar to the Tu *et al*. (2018) who showed the inhibitory effect of several plant essential oils at 1/2 MIC and MIC when testing with *E. coli*, *Salmonella typhimurium*, *B. subtilis*, and *S. aureus*.

The present study demonstrated the first time of herbal extracts could inhibit the growth of MAR–*A. veronii* isolated from the diseased Nile tilapia. The most effective of the antibacterial activity was observed in the extract of *C. sappan*. Our findings provided the important information of the potent herbal

extract that could be applied in modern aquaculture which favors using effective natural product instead of antibiotics to contol the bacterial disease. However, the adminitrarion route and appropriate dose of the herbal extract to effectively control the diseases has to be further investigated.

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