ORIGINAL ARTICLE

Time-kill kinetics and antimicrobial activities of Thai medical plant extracts against fish pathogenic bacteria

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ABSTRACT

The main objective of this work was to conduct the microbial control of Thai herbs against fish pathogens and their time-kill kinetics activity. Ten medicinal plants were selected to test antimicrobial activity against aquatic pathogens including *Aeromonas hydrophila*, *Flavobacterium* sp., and *Streptomyces* sp. *Caesalpinia sappan* and *Alpinia galangal* extracts showed the best activity against *A. hydrophila* and *Streptomyces* sp. Among them, *Caesalpinia sappan* expressed the great activity against *A. hydrophila* and *Streptomyces* sp. with the test concentration of MIC values of 1.25 and 2.50 mg/mL and MBC values of 5.0 and 10.0 mg/mL, while the MIC and MBC values of *A. galangal* were found to be 2.50 and 10.0 mg/mL with *Streptomyces* sp. The plant extracts of *C. sappan* and *A. galangal* at 1MIC, 2MIC, and 3MIC values really showed time-kill kinetics potential against fish pathogen on period of 3–18 h. In conclusion, plant extracts are good potentials sources as antifish pathogens and safety in an aquatic ecosystem.

Key words: Antimicrobial activity, fish pathogens, medicinal plants, time-kill kinetics

INTRODUCTION

Aquaculture is also a natural source for food industry. An increasing of aquaculture production for food supply forces the fish workers to push up fish communities and densities. Therefore, the increase of fish population leading to water quality, lack of sanitary management of aquaculture, and immune suppression that open out to disease infections such as viral and bacterial infections.[1,2] Bacteria is an important fish pathogen causing mortality and productivity, economic losses in aquaculture.[3] *Aeromonas hydrophila* is the major bacterial pathogen which found in freshwater[4] and associated with skin infection,[5,6] bacteremia, enteritis,[7] respiratory tract failure, and dysentery.[8] *Flavobacterium columnare* is found in freshwater and soil. It is very dangerous to fish baby that appearing skin problem as well as increasing mortality and related with poor environmental condition.[9,10] *Streptomyces* sp. is a family of actinomycetes, nonpathogenic bacteria and produce desiccation-resistant spores. Previously, actinomycetes have also shown interesting role in dealing with the fish bacterial diseases.[11]

Currently, the standard treatments for bacterial infection are antibiotics, vaccines, and chemical treatments. However, these treatments have limitation for the use, side effect, low efficacy, and antibiotic-resistant bacteria.[12] Therefore, different plant extracts can be potential alternative therapeutics to control fish pathogens. Plant extracts have alkaloids, flavonoids, steroids, and phenolic compounds.[13,14] Previous researches showed that bioactive compounds have antimicrobial.[15] In

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our study, we investigate the antimicrobial activity of plant extracts including Galangal (*Alpinia galanga* (L) Willd); AG, ginger (*Zingiber officinale* Roscoe; ZO), sappan (*Caesalpinia sappan* L.; CS), clove basil (*Ocimum gratissimum*; OG), red basil (*Ocimum sanctum* L.; OS), cassod tree (*Senna siamea* (Lam) Irwin and Barneby; SS), ringworm bush (*Cassia alata* (L) Roxb; CA), coriander root (*Coriandrum sativum* L.; CSA), garlic chives (*Allium tuberosum* Rottl. ex Spreng; AT), and cinnamon tree (*Cinnamomum verum* J. Presl.; CV) against *A. hydrophila*, *Flavobacterium* sp. and *Streptomyces* sp. and study the time-kinetics studies of herbal extracts at different times.

MATERIALS AND METHODS

Preparation of herbal plants

Ten plant samples (two rhizomes of ZO and AG, leaves of OG, SS, CA, and CSA, two stems of OS and AT, one core of CS, and one bark of CV) were collected from Pathum Thani province in May 2020 and identify the named by plant taxonomist (Miss Kunthasaya Akkarasiritharattana). All plants were taken in sterile container and transported to the laboratory, washed with running tap, and dried at 60°C for 1 day. Dried plants were ground and kept in sterile bottles under 4°C.

Extraction

The plant powders were extracted with 95% ethanol (1:20, w/v). The solution was shaken for 2 days at room temperature and filtered with filter paper (Whatman No. 1). All extraction were vaporized at 50°C, weighted, calculated, and stored at 4°C.

Antimicrobial activity

The antibacterial activity was examined by agar disc diffusion assay. Tested bacterial cultures including *A. hydrophila*, *Favobacterium* sp. and *Streptomyces* sp. were adjusted to 0.5 McFarland standards. Sterile discs were added with crude plant extracts (10 mg/mL) before applied over the agar plates which swabbed with tested bacteria. After incubation at 37°C for 1 day, the clear zone was observed. Gentamycin (10 µg/disc) and DMSO at 5% were used as control, respectively. The experiment was conducted in triplicates.[16]

Bacteriostatic and bactericidal activity

The bacteriostatic (MIC) of the plant extracts was examined by varying the concentrations between 0.1 and 10.0 mg/mL. For MIC, assay was performed in 96-well dilution method. Eighty milliliters of plant extracts with each concentration were added into each well that containing 100 µL of NB. After that, 20 µL of bacterial suspension (1.5×10^8 cfu/mL) was transferred into a 96-well plate. After incubation, the smallest concentration of plant extracts that inhibited the bacterial growth was evaluated by observing–measuring turbidity, called MIC values. To evaluate the MBC, the wells which did not present any visible growth (clear solution) were collected from each well using an inoculation loop and resubculture on NA. After incubation, the smallest concentration that no visible bacterial growth was noted as the MBC values. The assays were done in triplicate.[17]

Time-kill kinetic analysis

Time-kill kinetics assay was analyzed using the MIC values, which evaluated before the experiment. The test strain in late logarithmic growth phase was diluted the bacterial concentration in a serial ten-fold dilutions ranging of 10^{-3} and 10^{-4} from 1.5×10^8 cfu/mL. Eighty microliters of the extract at 1xMIC, 2xMIC, and 3xMIC was put into each well that containing 100 µL of NB supplemented with 20 µL of each diluted bacterial cells suspension and incubated at 37°C. Hundred milliliters of aliquot were taken from the mixture solution at 3, 6, 9, and 18 h and spread onto NA and then incubated at the same

Table 1: The inhibition zone of plant extracts against fish pathogens

Plant extracts	Origin	Family	% Yield	Inhibition zone (mm)		
				AHa	STb	FBc
AG	Rhizome	Zingiberaceae	7.00	NZf	13.83±0.76	NZ
CS	Core	Leguminosae	2.77	14.50±0.50	12.50±0.87	NZ
CC	Leaf	Labiatae	6.03	NZ	NZ	NZ
OT	Whole	Labiatae	1.40	NZ	NZ	NZ
SS	Leaf	Leguminosae	6.45	NZ	NZ	NZ
CA	Leaf	Caesalpinoideae	1.53	NZ	NZ	NZ
CSA	Root	Apiaceae	3.60	NZ	NZ	NZ
AS	Whole	Apiaceae	16.63	NZ	NZ	NZ
CV	Bark	Lauraceae	12.73	NZ	NZ	NZ
ZO	Rhizome	Zingiberaceae	6.50	NZ	NZ	NZ
+ve.[d]				14±0.00	13±0.00	13±0.00
-ve[e]				NZ	NZ	NZ

[a]*A. hydrophila*, [b]*Streptomyces* sp., [c]*Flavobacterium* sp., [d]gentamycin (10 µg/disc), [e]DMSO (5%), [f]no inhibition zone

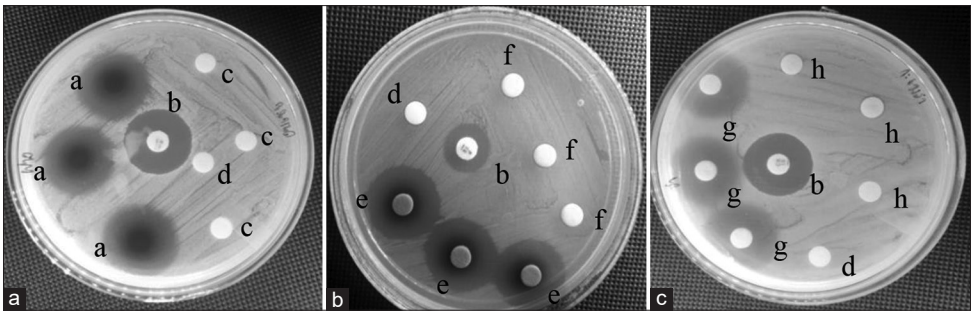

Figure 1: The inhibition zone of plant extracts against fish pathogens. (a) [a]CS extracts and [c]cinnamon extracts against *A. hydrophila,* (b) [c]CS extracts and [f]CC extracts against *Streptomyces* sp., (c) [g]AG extracts and [h]CC extracts against *Streptomyces* sp. [b]gentamycin, [d]5%DMSO

Table 2: The Bacteriostatic and bactericidal activity of plant extracts against fish pathogens

Plant extracts (mg/ml)	AH[a]		ST[b]	
	MIC	MBC[c]	MIC	MBC
CS	1.25	5.00	2.50	10.00
AG	-	-	2.50	10.00

[a]*A. hydrophila*, [b]*Streptomyces* sp.

condition. The colonies were counted and compared with control in terms of cfu/mL.[18]

RESULTS AND DISCUSSION

Yield extracts

The yield of plant extracts is demonstrated in Table 1. Data indicated that the yield content of AT has the highest levels at 16.63% followed by CV was 12.73%, whereas the crude extract of CA could only extract of 1.53% [Table 1]. An experimental is manage to obtain crude extracts with high product and lower change to the characteristics of crude plant extracts.[19] Some scientist documented that the influence of different plants and the type of residue on extraction yield were more essential than the solvent system on extraction yield.[20] Therefore, the selection of suitable extraction technique and solvent was considered.[21,22]

Antibacterial activity

The CS extracts (10 mg/mL) showed the most effective in reducing the growth of *A. hydrophila* and *Streptomyces* sp. with the zone of inhibition of 14.5 ± 0.50 and 12.5 ± 0.87 mm, while the rhizome extracts of AG were potent antimicrobial activity against *Streptomyces* sp. with the clearing zones of 13.83 ± 0.76 mm [Table 1 and Figure 1]. Several studies have demonstrated that CS has potential against broad bacterial strains as well as fungal pathogens (*Candida albicans* and *Aspergillus niger*).[23,24] Moreover, the *A. galanga* extracts have been showed against bacterial pathogens such as gastrointestinal tract pathogens, respiratory tract pathogens, and skin pathogens.[25]

Bacteriostatic and bactericidal activity

Previous results, the ethanolic extracts of CS demonstrated

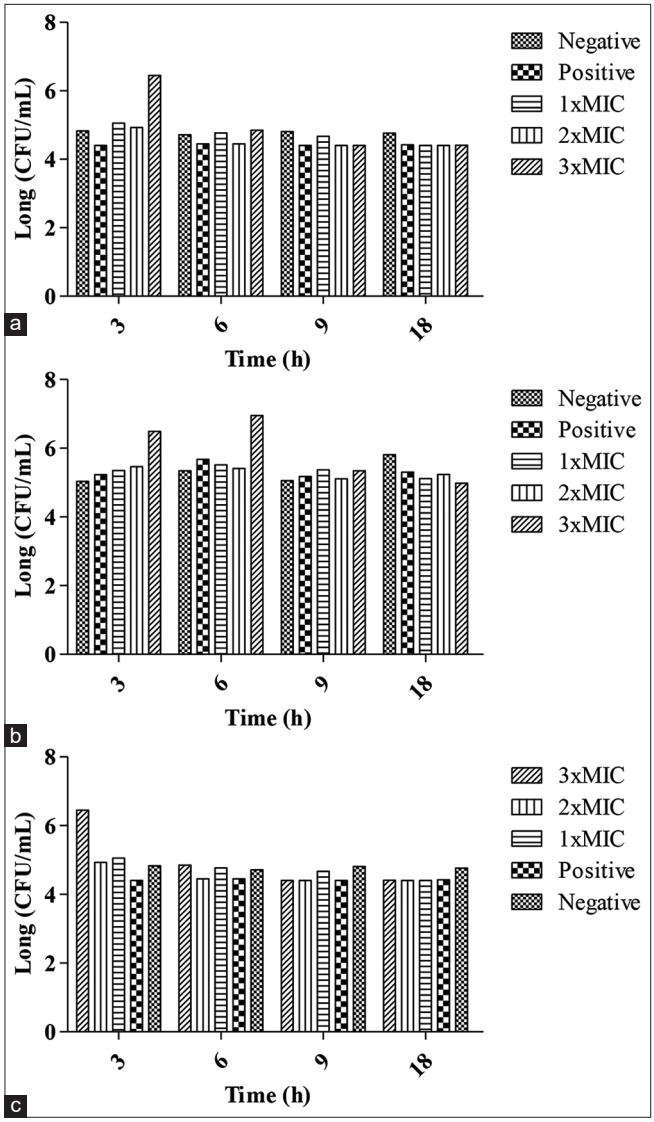

Figure 2: Time-kill kinetics of various concentration of plant extracts against fish pathogens. (a) AG: *Streptomyces* sp., (b) CS: *Streptomyces* sp., (c) CS: *Aeromonas hydrophila*

the antibacterial activity against *A. hydrophila* and *Streptomyces* sp., whereas the AG extracts had potent inhibitory effect against *Streptomyces* only. Therefore, this experiment was aimed to investigate MIC and MBC of both extracts (CS

and AG) against *A. hydrophila* and *Streptomyces* sp. Results displayed that the CS extracts exhibited antibacterial activity against *A. hydrophila* and *Streptomyces* with the MIC and MBC values of 1.25 and 2.50 mg/mL, and also represented the concentration of 5.0 and 10.0 mg/mL (MBC values), while the concentration of AG extracts at 2.50 and 10.0 mg/mL showed MIC and MBC activity with *Streptomyces* sp. [Table 2]. This experiment similar with some reported because the ethanol and water extracts of CS had an inhibitory effect against bacterial pathogens.[26] In addition, Voravuthikunchai *et al*.[27] noted that the MBC values of AG extracts against *S. aureus*, in contrast with the present report showed that the MBC of AG extracts against *Streptomyces* sp. was 10 mg/mL. Accordingly, variation in MIC and MBC values of different plant extracts may arise from variation in their method of extraction, tested organisms, feed inoculum, growth condition, and culture media that was used and bioactive compounds that were found in plant extract.[28,29]

Time-killing kinetics

To evaluate time-killing activity, this assay was performed over a period of 18 h with both fish bacteria and crude extracts at the concentration of 1xMIC, 2xMIC, and 3xMIC, respectively. As a result, time-kill curve was plotted between the logarithmic number of cfu/mL and incubation time. At 3xMIC concentration, CS extracts greatly showed decrease in amount of viable *A. hydrophila* and *Streptomyces* sp. at 9–18 h. In addition, AG extracts at the concentration of 2xMIC and 3xMIC could inhibit the bacterial cell viability of *Streptomyces* sp. at 9–18 h when compared with the 1xMIC and the negative control, respectively [Figure 2]. From the time, killing analysis exposed the degree of time-dependent microbial inhibition that was different between bacteria and plant extracts.[29,30] Accordingly, the ability of antibacterial properties may be considered by plant secondary metabolites and response to microbial infection.[31]

CONCLUSION

C. sappan and *A. galangal* seem to be the most promising medicinal plant for control fish pathogens disease. Therefore, further study is needed to test synergistic interaction between plant extracts and commercial agents as well as chemical properties and pharmacological activities.

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Conflicts of interest

There are no conflicts of interest.

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