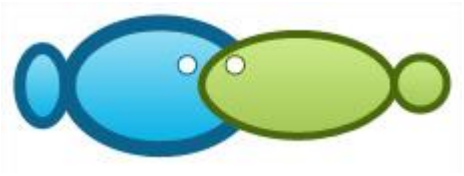

Effects of antibiotics and medicinal plants extracts against *Aeromonas hydrophila* isolated from *Rana rugulosa* in Thailand

[1]Chutharat Kanchan, [2]Kwanprasert Panchai, [1]Puttachat Imjai, [1]Leklai Chantabut, [3]Nukoon Kanchan, [4]Aonanong Chaiyara

[1] Faculty of Agricultural Technology, Rajabhat Maha Sarakham University, Maha Sarakham, Thailand; [2] Biology Program, Faculty of Science and Technology, Nakhon Ratchasima Rajabhat University, Thailand; [3] Animal Production Technology Program, Faculty of Agricultural Technology, Kalasin University, Thailand; [4] Fisheries Program, Faculty of Agricultural Technology, Sakon Nakhon Rajabhat University, Sakon Nakhon, Thailand. Corresponding author: C. Kanchan, chutharat.ka@rmu.ac.th

Abstract. Lowland frog (*Rana rugulosa*) is one of the commercial amphibian species that is becoming a popular-cultured species nowadays. *Aeromonas hydrophila* is an important bacterial pathogen which is commonly found in cultured frog. Frog diseases caused by *A. hydrophila* showed clinical signs such as red legs with hemorrhages, clouded eyes, ascites due to accumulation of fluids, diffuse white nodules in livers, spleens and kidneys and pale livers. *A. hydrophila* showed sensitivity to sulphamethoxazole/trimethoprim, followed by norfloxacin and trimethoprim, and two herbal extracts, namely *Piper sarmentosum* and *Terminalia catappa*. The minimum bactericidal concentration of two extracts ranged from 25 to 100 mg L^{-1} which could kill all *A. hydrophila*. The drug susceptibility showed the highest sensitivity at 22% sulphamethoxazole/trimethoprim and 16.67% of norfloxacin and trimethoprim. Hence, frog disease caused by *A. hydrophila* was actively controlled using sulphamethoxazole/trimethoprim and two extracts of *P. sarmentosum* and *T. catappa*.
Key Words: bacterial infection, drug susceptibility, frog disease, GC-MS, bioactive compounds.

Introduction. Lowland frog (*Rana rugulosa*) is one of the commercial amphibian species that is becoming popularly cultured nowadays in Thailand. One of the issues of frog cultivation is the infectious pathogens during rearing frogs in farms. Infectious pathogens such as fungus, parasite, bacteria and virus have been reported in captive and wild frogs, for example chytridiomycosis caused by *Batrachochytrium dendrobatidis* (Mazzoni 2003), dermatosepticemia caused by *Aeromonas hydrophila*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (Schadich & Cole 2010) and ranavirosis caused by *Ranavirus* (Campbell et al 2019). These pathogens cause morbidity or even mortality and induce significant economic losses in the frog populations. *Aeromonas hydrophila* is a gram-negative bacterium which produces significant disease in frogs, called red leg diseases or dermatosepticemia (Schadich & Cole 2010; Densmore & Green 2007; Huys et al 2003; Taylor et al 2001), as well as frog eggs (Khalifa et al 2020). The gross findings of *A. hydrophila* infection in frogs are hemorrhages, anorexia, swelling, edema, coelomic effusions and epidermal erosions, ulcers, sloughing or necrosis (Densmore & Green 2007). Antimicrobial drugs are commonly used in frog for bacterial disease treatment. There have been reported types of antibiotics including nitrofurantoin and oxytetracycline, which can suppress *A. hydrophila* isolated from frogs (Chinabut 1997), and also enrofloxacin, metronidazole, tetracycline and trimethoprim-sulfa (Vosjoli 2012). Unfortunately, disadvantages of antibiotic application were also reported, such as: induced bacteria resistant to antibiotic, accumulation of antibiotic residue in meat or environment and antibiotic resistant genes (Manaia et al 2016). Testing susceptibility of antibiotics to pathogens can reduce antibiotic use and improve an appropriate therapy

(Kerremans et al 2008). An alternative application was discovered: medicinal plants extracts against the frog pathogens. The present study aimed to determine the antibiotics as well as medicinal plants extracts efficacy against the *A. hydrophila* isolated from a deceased frog, farmed in Maha Sarakham Province, Thailand.

Material and Method

Collected specimen. The frog disease caused by *A. hydrophila* was collected from six frogs farmed in three districts of Maha Sarakham Province, in Thailand, during November 2019–July 2020. Both internal and external characteristics of the lesions were observed. The samples were necropsied and the abnormal organs such as livers, spleens and kidneys were isolated into tryptic soy agar and incubated at 25°C for 24 hours. The purified bacterium was identified by biochemical tests according to Buchanan & Gibbon (1974) and using API 20 E (BioMérieux, France).

Determination of the antibiotic susceptibility test. Eighteen strains of *A. hydrophila* derived from deceased farmed frogs were tested against fifteen antibiotics by the disc-diffusion method, including ampicillin (AMP10), amoxycillin/clavulanic acid (AMC30), ciprofloxacin (CIP5), chloramphenicol (C30), enrofloxacin (ENR5), erythromycin (E15), gentamicin (CN10), kanamycin (K30), norfloxacin (NOR10), novobiocin (NV30), oxytetracycline (OT30), oxolinic acid (OA2), sulphamethoxazole/trimethoprim (SXT25), tetracycline (TE30) and trimethoprim (W5) (Oxoid). Eighteen strains were triplicated and the zone diameter evaluation was performed, resulting a classification as susceptible, intermediate or resistant, according to criteria described by the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute 2012).

Determination of the medicinal plants extract susceptibility test. Ten medicinal plants extracts were investigated, the minimum inhibitory concentration (MIC) and minimun bactericidal concentration (MBC) against eighteen strains of *A. hydrophila*. The herbal extracts including *Azadirachta indica*, *Centella asiatica*, *Clitoria ternatea*, *Dolichandrone serrulata*, *Garcinia mangostana*, *Mangifera indica*, *Morinda citrifolia*, *Piper sarmentosum*, *Phyllanthus urinaria* and *Terminalia catappa* were prepared according to a modified technique of Ponnusamy et al (2010) and of Kanchan et al (2019). The crude ethanolic extracts were dissolved in dimethyl sulfoxide. The MIC and MBC were evaluated at a concentration of 200–1,563 mg mL^{-1} according to Kanchan et al (2019).

GC-MS analysis of two active crude extracts. Two crude extracts that showed high antimicrobial susceptibility were chosen for bioactive compounds analysis by gas chromatography-mass spectrometry (GC-MS) (Bruker). Briefly, 100 mg of crude extracts were dissolved in 1 mL of absolute ethanol then mixed by a vortex mixer and filtrated through 0.45 µm filter paper. A sample of 2 µL was injected with an auto sampler. The injector temperature was set at 250°C and the oven temperature was programmed from 40°C to 280°C. Mass spectra ranged between 35 and 550 amu with an electron impact ionization energy of 70 eV. The chemical components from the plants extracts were identified by comparing the retention times of chromatographic peaks and % area by using the National Institute of Standards and Technology (NIST) Mass spectral library 2008 database.

Results. The diseased frog with bacterial infection showed clinical signs such as lethargy, red legs with hemorrhages, ulcerations of the skin, clouded eyes, ascites due to the accumulation of fluids, diffuse white nodules in livers, spleens and kidneys and pale livers (Figure 1). The bacterial identification from the biochemical characteristics indicated the presence of *A. hydrophila*.

Antibiotic susceptibility test. *A. hydrophila* strains were found susceptible to chloramphenicol (61.11%), sulphamethoxazole/trimethoprim (22%), norfloxacin and trimethoprim (16.67%), oxytetracycline and tetracycline (11.11%), and ciprofloxacin,

enrofloxacin and kanamycin (5.56%) but resistant to ampicillin, amoxycillin/clavulanic acid, erythromycin, gentamicin, novobiocin and oxolinic acid (Figure 2). However, chloramphenicol was forbidden in aquaculture. Therefore, the remaining most effective antibiotic against *A. hydrophila* was sulphamethoxazole/trimethoprim, subsequently with norfloxacin and trimethoprim.

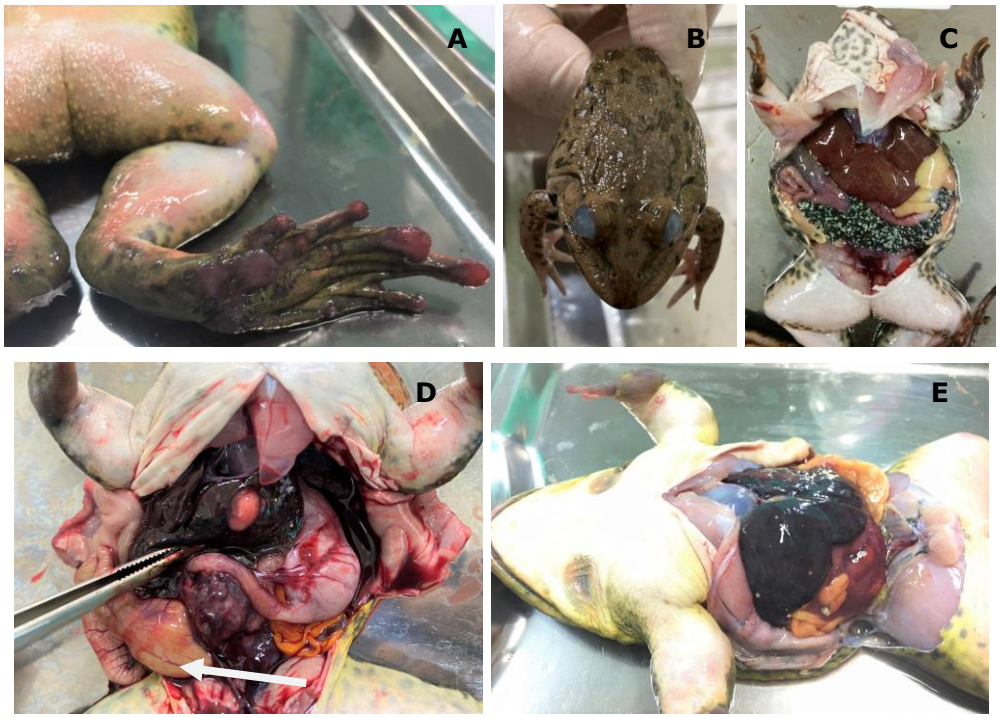

Figure 1. Gross pathology of diseased frog caused by *Aeromonas hydrophila* such as red leg (A), clouded eyes (B), pale liver (C), white nodules in spleen (D)(arrow), and white nodules in liver (D & E) (original).

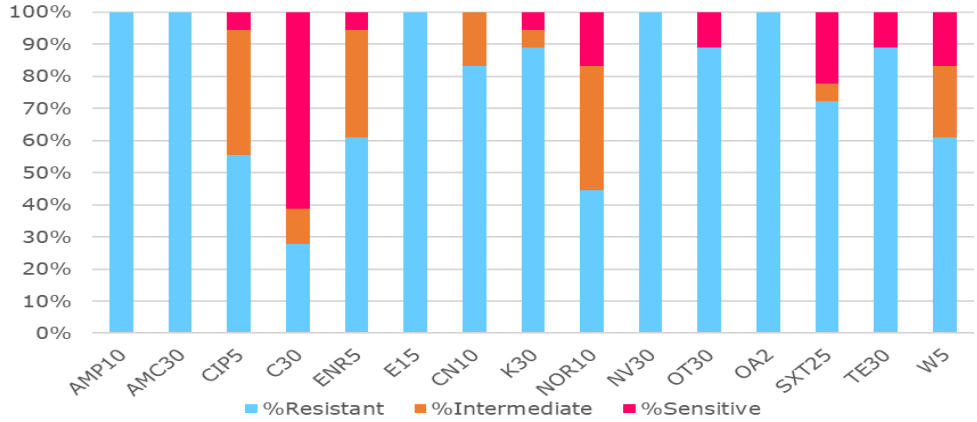

Figure 2. Antibiotic susceptibility test of eighteen *Aeromonas hydrophila* against 15 antibiotics: ampicillin (AMP10), amoxycillin/clavulanic acid (AMC30), ciprofloxacin (CIP5), chloramphenicol (C30), enrofloxacin (ENR5), erythromycin (E15), gentamicin (CN10), kanamycin (K30), norfloxacin (NOR10), novobiocin (NV30), oxytetracycline (OT30), oxolinic acid (OA2), sulphamethoxazole/trimethoprim (SXT25), tetracycline (TE30) and trimethoprim (W5).

Medicinal plants extract susceptibility test. The minimum inhibitory concentration (MIC) of ten herbal extracts ranged from 25 to more than 200 mg L^{-1}. The minimum bactericidal concentration (MBC) ranged from 12.5 to more than 200 mg L^{-1}. The highest antimicrobial effects observed in the leaves extracts were found in: (1) *P. sarmentosum*,

against all bacteria strains, with an MBC of 12.5 to 100 mg L^{-1}, (2) *T. cattapa*, with an MBC of 25 to 100 mg L^{-1}, (3) *D. serrulata*, with an MBC of 12.5 to more than 200 mg L^{-1}, (4) *M. indica*, and *G. mangostana* (peel extracts), with an MBC of 25 to more than 200 mg L^{-1}, (5) *C. asiatica*, *P. urinaria* and *C. ternatea* (flower extracts), with an MBC of 100 to more than 200 mg L^{-1}. The lowest antimicrobial effect was observed in leaves extracts of *A. indica*, at an MBC of 200 to more than 200 mg L^{-1} (Table 1).

Table 1

The minimum bactericidal concentration (MBC) of ten medicinal plants extract against eighteen strains of *Aeromonas hydrophila*

Medicinal plant extracts	*MBC (mg L^{-1})*						
	›200	*200*	*100*	*50*	*25*	*12.5*	*1.563*
Azadirachta indica	3 (16.67)	15 (83.33)	0	0	0	0	0
Centella asiatica	3 (16.67)	1 (5.56)	14 (77.78)	0	0	0	0
Clitoria ternatea	2 (11.11)	1 (5.56)	15 (83.33)	0	0	0	0
Dolichandrone serrulata	2 (11.11)	1 (5.56)	4 (22.22)	1 (5.56)	9 (50)	1 (5.56)	0
Garcinia mangostana	1 (5.56)	1 (5.56)	12 (66.67)	1 (5.56)	3 (16.67)	0	0
Mangifera indica	3 (16.67)	1 (5.56)	9 (50)	3 (16.67)	2 (11.11)	0	0
Morinda citrifolia	1 (5.56)	1 (5.56)	15 (83.33)	1 (5.56)	0	0	0
Piper sarmentosum	0	0	2 (11.11)	1 (5.56)	2 (11.11)	13 (72.22)	0
Phyllanthus urinaria	3 (16.67)	1 (5.56)	14 (77.78)	0	0	0	0
Terminalia catappa	0	0	1 (5.56)	11 (61.11)	6 (33.33)	0	0

Chemical constituents of P. sarmentosum and T. catappa crude extracts. Two crude extracts were analyzed by gas chromatography mass spectrometry (GC/MS) and the chemical constituents were identified based on their retention times of chromatographic peaks, area and % area, by using the NIST mass spectral library 2008 database (Figure 3, Figure 4). GC/MS analysis demonstrated 48 constituents in *P. sarmentosum* extracts (Table 2) and 26 constituents in *T. catappa* extracts (Table 3). Among the *P. sarmentosum* compounds, there were identified: benzenepropanoic acid, as the main component (23.07%), followed by the linoleic acid (18.83%), n-hexadecanoic acid (18.09%) and glycerin (6.83%) in. Among the *T. catappa* compounds, there were identified: the 1,2,3-benzenetriol as the major component (34.85%), followed by the n-hexadecanoic acid (15.43%), gamma-sitosterol (7.22%) and squalene (6.00%).

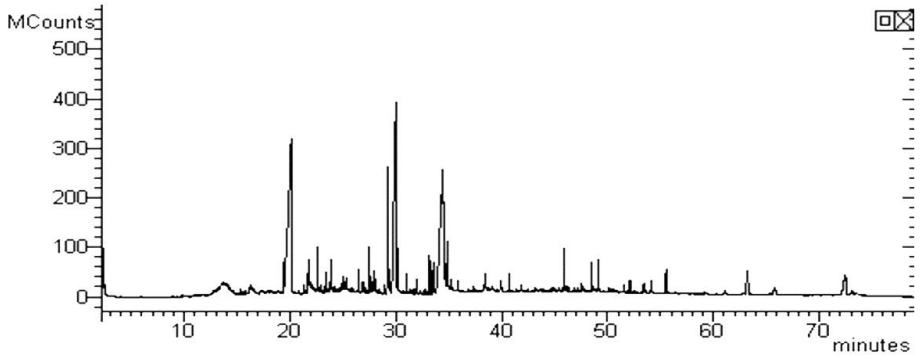

Figure 3. The GC-MS chromatogram of *Piper sarmentosum* extracts.

Table 2

Chemical composition of *Piper sarmentosum* extracts

RT (min)	*Peak name*	*Area (%)*
2.300	Acetic acid	1.92
13.694	Glycerin	6.83
19.398	Benzenepropanoic acid, ethyl ester	0.59
20.109	Benzenepropanoic acid	23.07
20.212	n-Decanoic acid	0.15
21.309	Cinnamic acid	0.40
22.608	Myristicin	0.81
23.246	Nerolidol	0.10
23.370	Dodecanoic acid	0.45
23.885	3-(4-Methoxyphenyl) propionic acid	1.22
24.971	beta Tumerone	0.29
25.287	Lauryl acrylate	0.21
26.472	Tetradecanoic acid	0.46
26.758	Loliolide	0.16
26.883	3,4-Dimethoxydihydrocinnamic acid	0.36
27.485	Phytol acetate	0.88
27.594	2-Pentadecanone, 6,10,14-trimethyl-	0.28
27.931	Pentadecanoic acid	0.43
28.125	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	0.26
28.864	Hexadecanoic acid, Methyl ester	0.14
29.263	Naphthalene, decahydro-1,1-dimethyl-	4.65
29.985	n-Hexadecanoic acid	18.09
30.174	Hexadecanoic acid, Ethyl ester	1.16
31.025	Farnesol	0.46
31.938	Oleic acid	0.60
33.165	Phytol	1.21
34.443	Linoleic acid	18.83
34.863	Octadecanoic acid	2.57
38.415	Cinnamic acid, isobutyl ester	0.70
39.935	2-Propenoic acid, 3-(2-formyl-4-methoxyphenyl)-, ethyl ester, (E)	0.39
40.682	Piperidine, 1-(3,4,5-trimethoxycinnamoyl)-	0.55
41.804	Glycerol beta-palmitate	0.21
45.892	Propionitrile, 3-(3,5-di-tert-butyl-4-hydroxyphenyl) thio-	1.32
46.807	Squalene	0.07
47.526	Pyrrolidine, 1-(1-oxo-11-octadecynyl)-	0.26
47.671	Piperyline	0.14
48.502	Methyl(Z) Cinnamate	0.79
49.139	Hexadecane, 1,16-dichloro-	0.81
50.157	gamma-Tocopherol	0.11
50.476	Cholesta-4,6-dien-3-ol, (3beta)-	0.11
51.552	dl-alpha-Tocopherol	0.22
52.106	Isosesamin	0.42
53.405	Campesterol	0.54
54.123	Stigmasterol	0.55
55.528	gamma-Sitosterol	1.17
63.223	Neophytadiene	1.80
65.777	Propanoic acid, 3,3'-thiobis-, didodecyl ester	0.63
72.430	Pentalene, octahydro-1-(2-octyldecyl)-	2.65
	Total	100.00

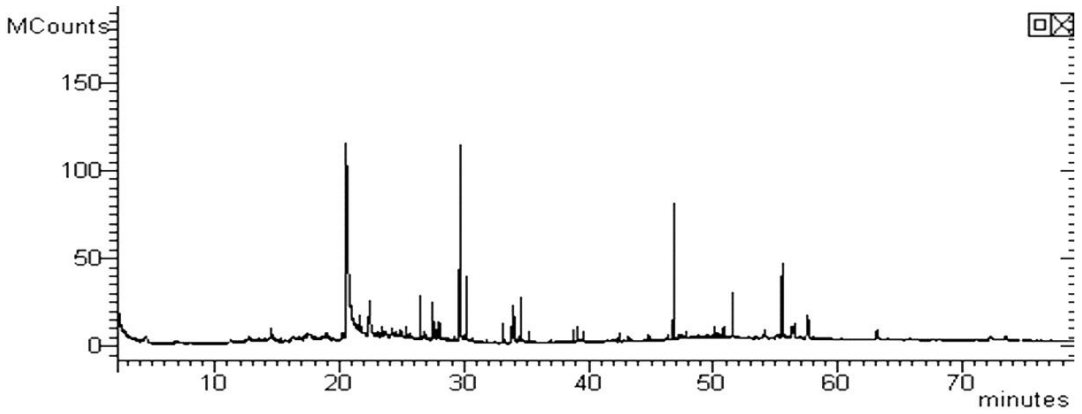

Figure 4. The GC-MS chromatogram of *Terminalia catappa* extracts extracts.

Table 3

Chemical composition of *Terminalia catappa* extracts

RT (min)	*Peak name*	*Area (%)*
14.475	Levoglucosenone	0.62
20.551	1,2,3-Benzenetriol	34.85
21.675	1-Dodecanol	0.66
22.435	beta-D-Glucopyranose, 1,6-anhydro-	5.27
26.443	Tetradecanoic acid	1.67
27.484	Phytol acetate	1.20
27.596	2-Pentadecanone, 6,10,14-trimethyl-	0.62
28.127	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	0.65
29.701	n-Hexadecanoic acid	15.43
30.159	Hexadecanoic acid, ethyl ester	2.90
33.148	Phytol	1.19
33.823	11,14-Eicosadienoic acid, methyl ester	1.25
33.960	trans-13-Octadecenoic acid	3.60
34.558	Octadecanoic acid	2.96
38.791	4,8,12,16-Tetramethylheptadecan-4-olide	0.64
39.077	Eicosanoic acid	0.99
39.591	Eicosanoic acid, ethyl ester	0.52
46.814	Squalene	6.00
50.149	gamma-Tocopherol	0.57
51.548	dl-alpha-Tocopherol	2.83
54.109	Stigmasterol	0.98
55.537	gamma-Sitosterol	7.22
56.319	Olean-12-ene	1.27
56.500	Olean-18-ene	1.51
57.588	Lupeol	3.19
63.150	Neophytadiene	1.41
	Total	100.00

Discussion. Aquaculture of *R. rugulosa* to provide frog's meat in Thailand has become a frequent practice in many countries of the Southeast Asia (Uppanunchai et al 2012), due to a high demand. The issues met during the frog farming are related to the infectious disease caused by various pathogens, including bacteria, fungi, parasites and viruses (Campbell et al 2019; Layla et al 2018; Schadich & Cole 2010; Mazzoni 2003). *A. hydrophila* is a major bacterial disease in amphibians, especially in captive and wild frogs (Schadich & Cole 2010). In the present study, the macroscopic lesions of the diseased Lowland frog externally revealed red legs with hemorrhages, clouded eyes and ascites due to accumulation of fluids. Additionally, internal characteristics were abnormally found, such as dissemination of white nodules in livers, spleens, kidneys and pale livers.

These results were similar to the study of Densmore & Green (2007), who reported *A. hydrophila* infection in frogs which revealed hemorrhages, swelling, edema, coelomic effusions, epidermal erosions and ulcers. Nevertheless, internal multiple white nodules in livers, spleens and kidneys were rarely reported in frog diseases caused by *A. hydrophila*. There have been reports related to white nodules in the internal organs of frog species, including the studies of: (1) Fremont-Rahl et al (2015), who described multiple nodular foci in the spleen and liver caused by *Mycobacterium liflandii* in *Xenopus* (*Silurana*) *tropicalis* frogs; (2) Ikuta et al (2018) and Haridy et al (2014), who discovered multiple white nodules in liver, spleen, heart, lungs, ovaries and kidneys of frog *Lithobates catesbeiana* and *Rhacophorus arboreus*, respectively and (3) Hosoya et al (2015), who revealed white nodules in the internal organs, due to fungal diseases, which is also the first report of *Veronaea botryosa* as a causal agent of chromomycosis in frogs.

The treatment of bacterial infection commonly uses antibiotics. In this study, the most effective authorized drug against *A. hydrophila*, based on the disc diffusion method, was the sulphamethoxazole/trimethoprim, followed by norfloxacin and trimethoprim. The strong antibiotic activity of the sulphamethoxazole/trimethoprim was corroborated by Vosjoli (2012), but *A. hydrophila* also showed a certain susceptibility to enrofloxacin and tetracycline. On the other hand, all *A. hydrophila* showed resistance to ampicillin, amoxycillin/clavulanic acid, erythromycin, gentamicin, novobiocin and oxolinic acid. This bacterial pathogen showed multidrug-resistant pattern, based on the susceptibility test.

In the present study, among ten medicinal extracts, only two extracts, the *P. sarmentosum* and *T. catappa*, had the best antibacterial activity against all eighteen strains of *A. hydrophila*. Nevertheless, none of the antibiotics were susceptible to all bacterial pathogens. This data was a good finding to discover the active medicinal plants extracts against the bacterial diseases caused by *A. hydrophila* in frog culture. Additionally, the benzenepropanoic acid was a major bioactive compound detected in *P. sarmentosum* leaves extracts. This compound has antifungal, antioxidant activities (Bashir et al 2012). The main compound present in the *T. catappa* leaves extracts was 1,2,3 benzene triol which exhibited antibacterial activity. This result was corroborated by the report of Mohammad et al (2015).

Conclusions. This study has documented the *A. hydrophila* infection in cultured *R. rugulosa* and its antimicrobial effects of both antibiotics and medicinal plants extracts. Sulphamethoxazole/trimethoprim and leaves extract of *P. sarmentosum* and *T. catappa* have a great antimicrobial activity against *A. hydrophila*. Benzenepropanoic acid and 1,2,3 benzene triol were the major compounds possessing an antimicrobial activity. Both medicinal plants extracts could be applied as an alternative antibacterial agent against *A. hydrophila* and are useful to reduce the use of antibiotic in order to address the issue of antibiotic-resistant bacteria in frog populations.

Acknowledgements. The authors would like to thank for the research grant of Rajabhat Maha Sarakham University.

Conflict of interest. The authors declare no conflict of interest.

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Received: 17 February 2021. Accepted: 26 April 2021. Published online: 08 May 2021.
Authors:
Chutharat Kanchan, Rajabhat Maha Sarakham University, Faculty of Agricultural Technology, 44000 Maha Sarakham, Thailand, e-mail: chutharat.ka@rmu.ac.th
Kwanprasert Panchai, Nakhon Ratchasima Rajabhat University, Biology Program, Faculty of Science and Technology, 30000 Thailand, e-mail: kwan.p@nrru.ac.th
Puttachat Imjai, Faculty of Agricultural Technology, Rajabhat Maha Sarakham University, 44000 Maha Sarakham, Thailand, e-mail: puttachat.im@rmu.ac.th
Leklai Chantabut, Rajabhat Maha Sarakham University, Faculty of Agricultural Technology, 44000 Maha Sarakham, Thailand, e-mail: jantabood@hotmail.com
Nukoon Kanchan, Kalasin University, Animal Production Technology Program, Faculty of Agricultural Technology, 46000 Thailand, e-mail: nukoon.ka@ksu.ac.th
Aonanong Chaiyara, Sakon Nakhon Rajabhat University, Fisheries Program, Faculty of Agricultural Technology, 47000 Sakon Nakhon, Thailand, e-mail: aonanong.c@snru.ac.th

How to cite this article:
Kanchan C., Panchai K., Imjai P., Chantabut L., Kanchan N., Chaiyara A., 2021 Effects of antibiotics and medicinal plants extracts against *Aeromonas hydrophila* isolated from *Rana rugulosa* in Thailand. AACL Bioflux 14(3):1222-1230.