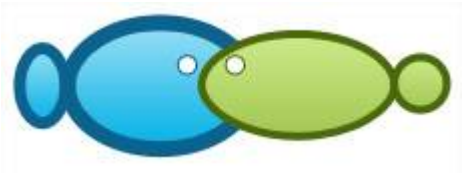

Synergistic effect of *Piper sarmentosum* and *Terminalia catappa* extracts against *Aeromonas hydrophila* infection in *Rana rugulosa*

[1]Chutharat Kanchan, [1]Puttachat Imjai, [1]Leklai Chantabut, [2]Kwanprasert Panchai, [3]Nukoon Kanchan, [4,5]Aonanong Khundech

[1] Agriculture Program, Faculty of Agricultural Technology, Rajabhat Maha Sarakham University, Maha Sarakham, 44000, Thailand; [2] Biology Program, Faculty of Science and Technology, Nakhon Ratchasima Rajabhat University, 30000, Thailand; [3] Department of Animal Production Technology, Faculty of Agricultural Technology, Kalasin University, 46000, Thailand; [4] Fisheries Program, Faculty of Agricultural Technology, Sakon Nakhon Rajabhat University, Sakon Nakhon, 47000, Thailand; [5] Aquatic Animal Production and Aquaculture Innovation Research Unit, Center of Excellence in Modern Agriculture, Sakon Nakhon Rajabhat University, Sakon Nakhon 47000, Thailand. Corresponding author: C. Kanchan, chutharat.ka@rmu.ac.th

Abstract. *Aeromonas hydrophila* infection is a significant etiological agent that causes red leg disease in cultured frogs. *In-vitro* trials have demonstrated the potent antibacterial activities of two herbal extracts, namely *Piper sarmentosum* and *Terminalia catappa*. Therefore, this study investigated the *in-vivo* effects of feeding frogs with a mixed herbal extract supplement in the presence of *A. hydrophila* challenge. The dietary supplementation of the mixed herbal extracts for 30 days significantly reduced mortality and enhanced disease resistance against *A. hydrophila* ($p < 0.05$). These findings suggest that the mixture of the two herbal extracts can be utilized as a dietary approach for the treatment of bacterial infections caused by *A. hydrophila*.

Key Words: *Aeromonas hydrophila*, chemical composition, lowland frog, treatment, mixed herbal. extracts.

Introduction. Frog production in aquaculture has gained significant market value, owing to the increasing global demand (FAO 2022). The world production of frog meat was estimated at 44,000 tons annually from 1999 to 2008, which subsequently increased to 85,000 tons in 2008 (FAO 2010). However, infectious diseases pose a major challenge, leading to economic losses in frog farming. Bacterial diseases, in particular, are significant concerns in the lowland frog (*Rana rugulosa*) culture. Among them, *Aeromonas hydrophila*, a pathogenic species, is reported to affect both aquatic animals (Hu et al 2012) and amphibians (Densmore & Green 2007; Schadich & Cole 2010). *A. hydrophila* displays a rod-shaped morphology and is characterized as Gram-negative, facultatively anaerobic, oxidase-positive, catalase-positive, capable of fermenting glucose, and devoid of spore formation (El-Sharaby et al 2021). Clinical signs of *A. hydrophila* infection in frog culture include red legs (Taylor et al 2001; Huys et al 2003; Densmore & Green 2007; Schadich & Cole 2010; Kanchan et al 2021), ascites or edema (Densmore & Green 2007; Kanchan et al 2021), clouded eyes, pale liver, and white nodules in internal organs (Kanchan et al 2021). Effective treatment strategies for *A. hydrophila* induced diseases are crucial to mitigate economic losses. Conventional antimicrobials such as enrofloxacin, tetracycline, and trimethoprim-sulfa are commonly used (de Vosjoli 2012). However, *A. hydrophila* has demonstrated multi-resistance to antibiotics, as indicated by its high minimum inhibitory concentration values (MIC) (Kanchan et al 2021). Therefore, alternative approaches are sought to address antibiotic-resistant bacteria, and plant extracts have garnered interest due to their production of

secondary metabolites or phytochemical compounds that exhibit inhibitory or bactericidal effects against pathogenic bacteria (Mulia et al 2022; Rangel-López et al 2022; Effendi et al 2023; Purbomartono et al 2023). Several studies have reported the potential of herbs for treating diseases in fish (Shakya 2017; Stratev et al 2018), but there is limited research on their application in frogs. Kanchan et al (2021) documented the antibacterial screening of ten medicinal plant extracts against *A. hydrophila* isolates. Among them, *Piper sarmentosum* and *Terminalia catappa* exhibited promising efficacy against *A. hydrophila* isolates from diseased frogs through *in vitro* broth dilution trials. This present study aims to investigate the treatment of *A. hydrophila* infection in frogs through dietary supplementation of mixed extracts from *P. sarmentosum* and *T. catappa*.

Material and Method. The experiment to investigate the efficiency of mixed herbal extracts against *A. hydrophila* was carried out from October 2021 to December 2021.

Experimental animal. The frogs used in this research had an average size of 88±2 g and were sourced from a private farm in Muang District, Maha Sarakham Province, Thailand. The frogs were kept at the farm of the fisheries program, Faculty of Agriculture, Rajabhat Maha Sarakham University, for 2 weeks before being utilized. Commercial diets were fed ad libitum twice a day (the application number for a license to use animals for scientific purposes was U1-04522-2559).

Preparation of experimental diets. Two extracts from medicinal plants were individually prepared following the methodology outlined by Kanchan et al (2019). Each herbal extract was then combined with commercial feed through spraying, using the desired concentration. Subsequently, the supplemented diets were air-dried at room temperature for one day prior to their utilization in the experiment.

Experimental design and challenge test. One hundred and fifty frogs were divided into five experimental groups. A bacterial solution of *A. hydrophila* was administered through intraperitoneal injection at a volume of 0.1 mL frog^{-1} in the treatment groups, namely groups I, II, III, and V. In contrast, group IV received an intraperitoneal injection of 0.1 mL frog^{-1} of 0.85% NaCl, serving as the negative control group without bacterial inoculation. Furthermore, the frogs were fed with commercial feed (without herbs) for 7 days after post-inoculation. Subsequently, dietary supplementation with a mixture of two herbal extracts (*P. sarmentosum* and *T. catappa*) was initiated as follows: group I contained a combination of both *P. sarmentosum* and *T. catappa* at a concentration of 12.5 mg mL^{-1} each; group II received 12.5 mg mL^{-1} of *P. sarmentosum* and 25 mg mL^{-1} of *T. catappa*; group III received 12.5 mg mL^{-1} of *P. sarmentosum* and 50 mg mL^{-1} of *T. catappa*; group IV served as the negative control, while group V served as the positive control and was fed with commercial feed (without herbs). The treatment trials were conducted over 30 days and were administered at 3% of the frogs' body weight. The mean cumulative mortality and survival rate in each group were monitored daily after post-injection for 30 days. All surviving frogs were subjected to external and internal observation for gross lesions at the end of the experiment. The data were analyzed using analysis of variance (ANOVA), and mean values were compared using Duncan's Multiple Range Tests with SPSS version 26 for Windows.

Chemical composition of the mixed P. sarmentosum and T. cattappa extracts. The chemical composition of the two mixed crude extracts was analyzed using gas chromatography-mass spectrometry (GC-MS) with a Bruker instrument. A total of 50 mg of the mixed extracts was dissolved in 1 mL of absolute ethanol and then filtered through a 0.45 µm filter paper. The methodology was conducted in accordance with Kanchan et al (2021). The chemical constituents of the mixed extracts were identified and compared with the National Institute of Standards and Technology (NIST) Mass Spectral Library 2008 database, based on their retention times and the peak areas of the chromatogram.

Results. The herbal treatment involved the combination of two extracts, namely *P. sarmentosum* and *T. catappa* extracts, which were assessed for their efficacy against *A. hydrophila* infection in *R. rugulosa*. The moribund frogs in the positive control group (group V), which were injected with bacteria, exhibited prominent clinical signs. These signs included red legs, skin lesions on the legs, leading to ascites due to fluid accumulation, enlarged gall bladders, and pale livers during the experimental period (Figure 1). Nevertheless, the treatment groups exhibited slight abnormal lesions, such as red legs in groups II and III. The clinical findings among the surviving frogs in the treatment group mainly comprised pale livers (Figure 2), along with slightly white nodules in the liver, spleen, and swollen kidneys. Furthermore, the treatment group that received the higher concentration of the combined extracts (groups II and III) exhibited paler livers than group I. The surviving frogs in the untreated group with bacterial injection (group V) exhibited similar internal findings as those described in the treatment group. Additionally, they displayed more abnormal characteristics, notably ascites due to fluid accumulation. The untreated frogs without bacterial injection (group IV) displayed normal characteristics.

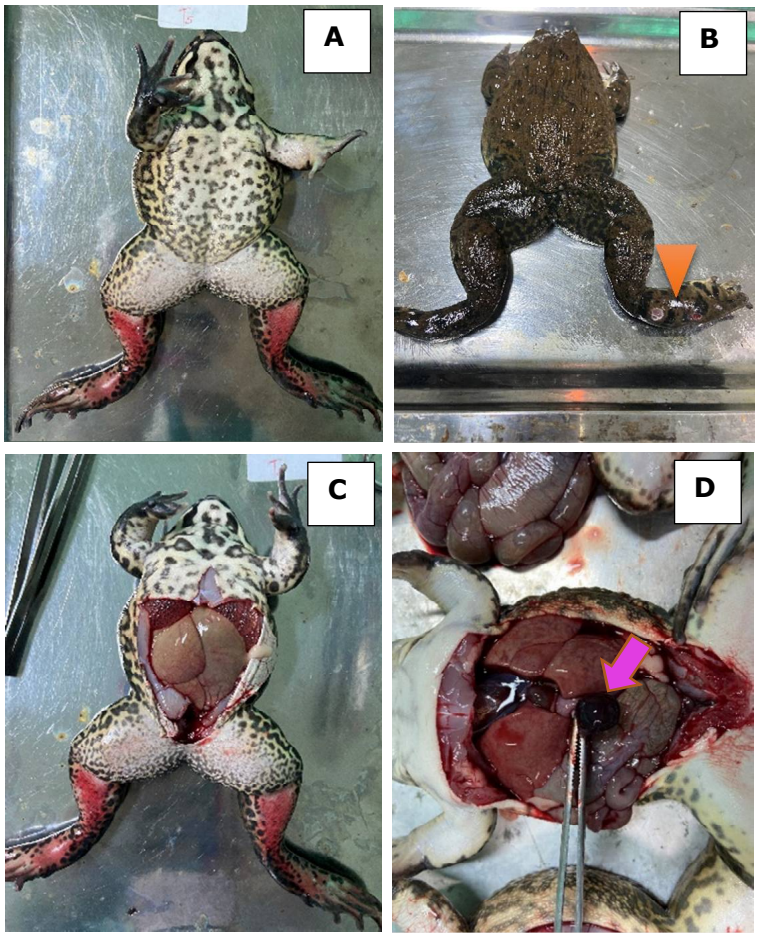

Figure 1. The untreated frog injected with bacteria (group V) exhibited clinical signs, including red legs (A), leg lesions (B) (arrowhead), pale livers (C), and enlarged gallbladders (D) (arrow).

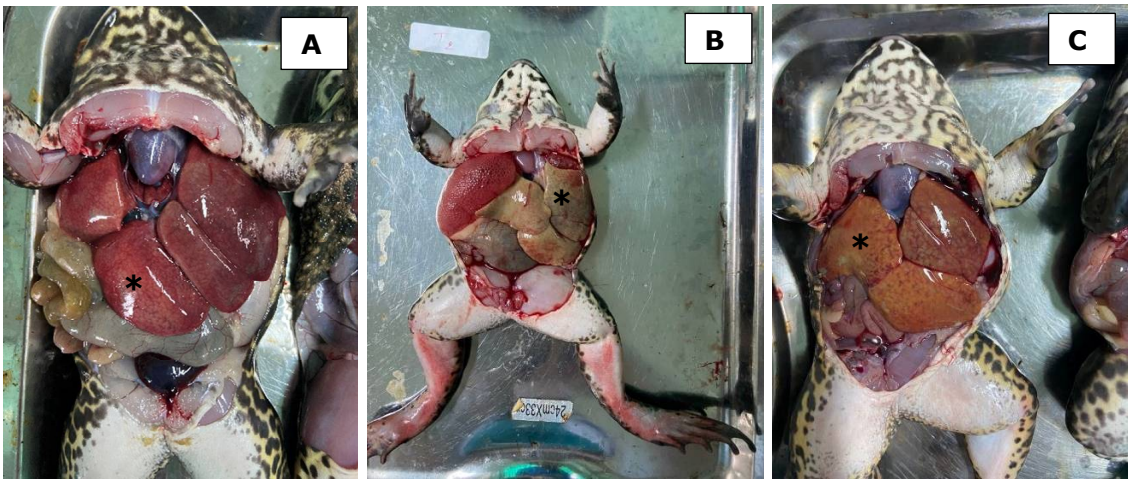

Figure 2. The surviving frogs in the treatment groups, namely group I (A), group II (B), and group III (C), exhibited primarily pale livers (*).

Mortality and survival rate. The dietary supplementation of a mixture of two herbal extracts for 30 days significantly reduced mortality and increased disease resistance in frogs following a challenge with 0.1 mL of 10^8 cfu mL^{-1} of *A. hydrophila* compared to the negative control group after post-infection. The mean cumulative mortality after post injection with *A. hydrophila* in the experimental group was 3.33±5.77, 16.67±5.77, 23.33±5.77, 0, and 56.67±5.77, respectively for groups I to V. Subsequently, the survival rates of the experimental groups were 96.67±5.77, 83.33±5.77, 76.67±5.77, 100, and 43.33±5.77, respectively (Figure 3). The concentration of the two mixed extracts in group I (12.5 mg mL^{-1} of *P. sarmentosum* and *T. catappa*) exhibited the lowest mortality compared to all post-injection treatments with the bacterium ($p < 0.05$). The results suggested that the dietary mixture of the two herbal extracts could be used for bacterial infection caused by *A. hydrophila*.

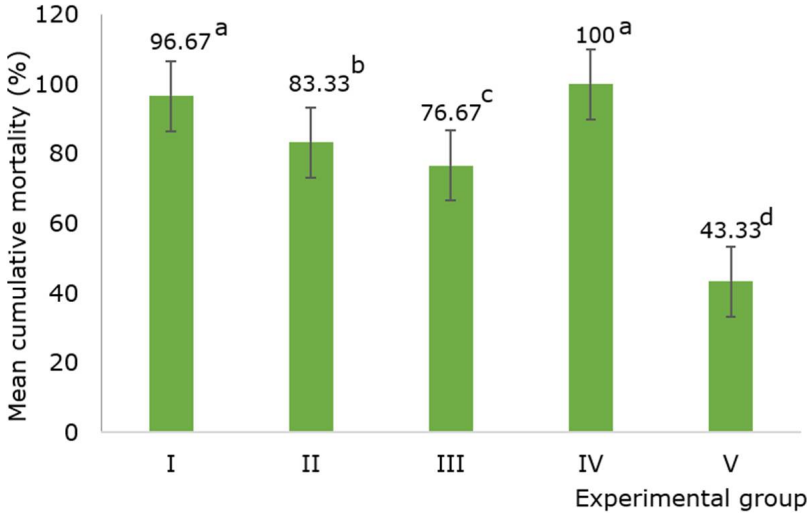

Figure 3. The mean survival rate of *R. rugulosa* after a 30-day treatment with a mixture of herbal extracts.

The mean survival rate of *R. rugulosa* was assessed after being exposed to with *A. hydrophila* and subsequent treatment with varying concentrations of mixed herbal extracts (*P. sarmentosum* and *T. catappa*). Group I received the lowest concentration, Group II the moderate concentration, and Group III the highest concentration. Both Group IV and Group V were provided with a herb-free commercial feed. Significant differences between the experimental groups are represented by lowercase letters ($p > 0.05$), as depicted in the Figure 3.

Chemical composition of mixed P. sarmentosum and T. cattappa extracts. Fifty-one chemical constituents were identified in the mixture of the two herbal extracts through GC-MS analysis (Figure 4, Table 1). The predominant bioactive compound was asarone (28.20%), followed by 1H-pyrrole, 1-(1-oxo-3-phenylpropyl) (25.71%), benzenepropanoic acid, ethyl ester (4.88%), and phytol (4.21%). However, the presence of beta-Asarone was only 0.76%.

Table 1

Chemical composition of mixed two herbal extracts

RT (min)	*Peak name*	*%Area*
11.482	beta-Linalool	0.06
12.976	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	0.11
15.441	4-Ethenylphenol	0.11
16.430	Benzenepropanoic acid, methyl ester	0.03
16.881	2-Undecanone	0.09
17.632	2-Methoxy-4-Vinylphenol	0.09
18.514	Benzenepropanoic acid, ethyl ester	4.88
19.539	beta-Elemene	0.51
19.901	Benzenepropanoic acid	1.72
20.309	beta-Caryophyllene	2.87
21.125	alpha-Caryophyllene	0.50
21.984	beta-Selinene	1.43
22.198	alpha-Selinene	1.52
22.321	Isoeugenol methyl ether	2.11
23.766	Nerolidol	0.46
24.305	Caryophyllene oxide	0.31
24.651	3-(4-Methoxyphenyl) propionic acid ethyl ester	0.47
25.153	beta-Asarone	0.76
25.864	Isoelemicin	0.87
26.821	Asarone	28.20
27.629	1H-Pyrrole, 1-(1-Oxo-3-Phenylpropyl)-	25.71
28.040	Aspidinol	0.10
29.687	Phytol acetate	0.74
29.795	2-Pentadecanone, 6,10,14-trimethyl-	0.12
30.528	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	0.23
31.556	4-(1-Phenethyl-1H-tetrazol-5-ylmethyl)-morpholine	0.62
31.945	(E,E,E)-3,7,11,15-Tetramethylhexadeca-1,3,6,10,14-pentaene	0.12
32.540	1-(Cyclopropylmethyl)-4-(methyloxy)benzene	1.05
32.802	Hexadecanoic acid, ethyl ester	0.28
33.683	Geranyl linalool isomer B	0.33
35.618	Pyrrolidine, 1-cinnamoyl-	0.38
36.030	Phytol	4.21
37.014	9,12-Octadecadienoic acid, ethyl ester	0.18
37.172	Linoleic acid ethyl ester	0.26
37.843	Octadecanoic acid, ethyl ester	0.28
38.351	Neophytadiene	0.07
41.046	Morpholine, 4-(1-oxo-3-phenyl-2-propenyl)-	0.46
42.401	Pryyolidine, 1-(m-methoxycinnamoyl)-	3.10
44.202	Glycerol beta-palmitate	0.49
45.916	4-Pentenoic acid, 2,2-diethyl-3-oxo-5-phenyl-, ethyl ester	0.69
48.017	[1,1'-Biphenyl]-4-Carbonitrile, 2'-(Methylamino)-	0.52
49.036	Squalene	1.88
52.119	beta-Tocopherol	0.14
52.369	gamma-Tocopherol	0.52
53.863	dl-alpha-Tocopherol	3.93
55.692	Campesterol	0.76
56.415	Stigmasterol	1.13
57.963	gamma-Sitosterol	2.93
58.612	beta-Amyrin	0.43
59.881	Lupeol	0.75
61.492	Vitamin E	0.52
	Total	100

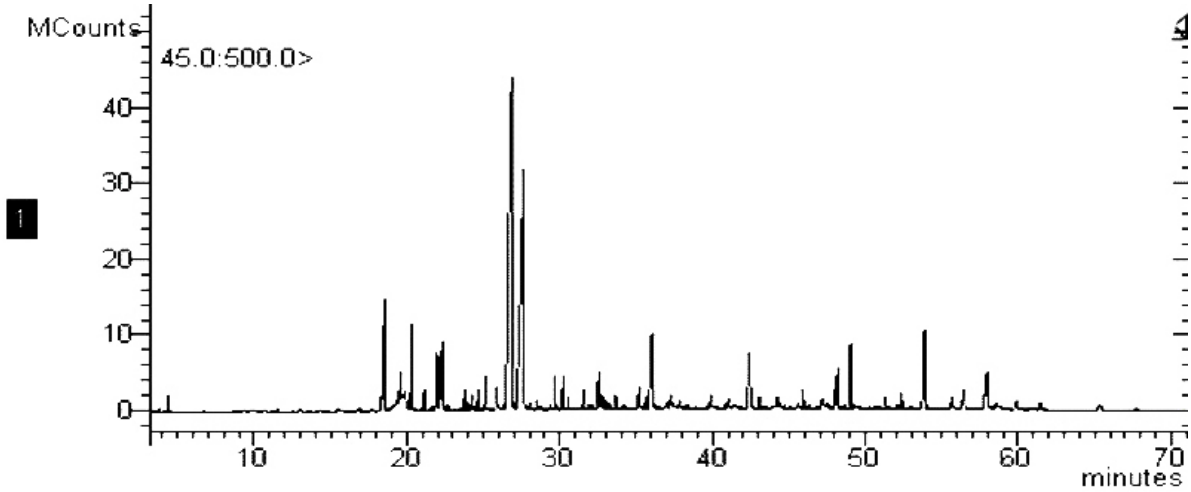

Figure 4. The GC-MS chromatogram of two mixed herbal extracts (*P. sarmentosum* and *T. catappa*).

Discussion. *A. hydrophila*, a notable pathogen, poses a significant menace as it is accountable for bacterial diseases in fish, reptiles, and amphibians (Dias et al 2016). Red leg disease, an outcome of *A. hydrophila* infection, manifests as conspicuous symptoms including hemorrhages, edema, coelomic effusions (Densmore & Green 2007), and ulcers (Densmore & Green 2007; Hill et al 2010) in frogs. In this study, the gross lesions observed in the positive control group (*A. hydrophila* injection without herb feeding) exhibited analogous characteristics as described by Densmore & Green (2007). The pathogenicity test serves as a mean to assess the virulence of bacterial pathogens towards their native hosts. Furthermore, following intraperitoneal injection, the cumulative mortality rate of *A. hydrophila* infection was remarkably high at 56.67± 5.77%. This contrasts with the findings of Schadich & Cole (2010), where no morbidity or mortality was observed in frogs exposed to *A. hydrophila* via bath exposure. The results obtained from treating bacterial infections caused by *A. hydrophila* using a combination of two mixed herbal extracts (*P. sarmentosum* and *T. catappa*) indicate the potential of herbal extracts in effectively combating such infections in frogs. The study revealed a noteworthy decrease in mortality rate among frogs treated with these extracts, thereby underscoring the efficacy of these medicinal plant extracts against bacterial infections. This observation highlights the synergistic action of the two extracts, resulting in a pronounced therapeutic effect. The two mixed herbal extracts primarily contain asarone, a compound renowned for its aromatic and medicinal properties. Extensive research has investigated the potential uses and therapeutic effects of asarone in traditional medicine systems (Das et al 2019). The next compound examined was 1H-pyrrole, 1-(1-oxo-3-phenylpropyl), which is classified as an alkaloid found in the fruit of *P. sarmentosum* (Buckingham et al 2010). Several studies on the properties of alkaloids have demonstrated their potential pharmacological effects, including antimicrobial activity trials against pathogens in aquatic animals (Cushnie et al 2014). Furthermore, Shakya (2017) emphasizes the emerging trend of employing medicinal plants as dietary supplements for the management of aquatic diseases and the promotion of overall health in aquatic animals. The present study further contributes to this trend, illustrating that the combined extracts of both herbs can serve as a viable alternative for the treatment of bacterial infections in frogs. This approach shows promise in potentially replacing the use of antibiotics, thereby not only enhancing disease management but also fostering the safe cultivation of frogs.

Conclusions. The study aimed to assess the efficacy of a combination of mixed herbal extracts derived from *P. sarmentosum* and *T. catappa* leaves in treating bacterial infections induced by *A. hydrophila* in Lowland frogs. Experimental frogs were orally administered varying concentrations of the mixed extracts for a duration of 30 days following injection with an *A. hydrophila* suspension (10^8 CFU mL^{-1}). Throughout the

experiment, the presence of external gross lesions such as red legs and leg lesions, as well as internal gross lesions including edema, swollen kidneys, white nodules in internal organs, and pale liver, was closely monitored. Results indicated that the experimental frogs treated with the mixed extracts exhibited a reduced incidence of abnormal symptoms in comparison to the positive control group (bacterial injection without herb feeding). The optimal concentrations for combating *A. hydrophila* infections were determined to be 12.5 mg mL^{-1} for both herb extracts. Notably, the mean cumulative mortality rate of the treated group was 3.33±5.77%, signifying a significant difference from the positive control group. Furthermore, the survival rate among frogs receiving herbal extracts at 12.5 mg mL^{-1} was measured to be 96.67±5.77%. In conclusion, these findings demonstrate the potential utilization of the combined extracts derived from *P. sarmentosum* and *T. catappa* leaves for effectively treating bacterial infections caused by *A. hydrophila* in *Rana rugulosa*.

Acknowledgements. We express our sincere gratitude to the Research Development Institute of Rajabhat Maha Sarakham University for their invaluable financial support towards this research project.

Conflict of interest. The authors declare that there is no conflict of interest.

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Received: 30 August 2023. Accepted: 27 September 2023. Published online: 06 October 2023.
Authors:
Chutharat Kanchan, Rajabhat Maha Sarakham University, Faculty of Agricultural Technology, 44000 Maha Sarakham, Thailand, e-mail: chutharat.ka@rmu.ac.th
Puttachat Imjai, Rajabhat Maha Sarakham University, Faculty of Agricultural Technology, 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
Kwanprasert Panchai, Nakhon Ratchasima Rajabhat University, Biology Program, Faculty of Science and Technology, 30000 Thailand, e-mail: kwanprasert.p@nrru.ac.th
Nukoon Kanchan, Kalasin University, Department of Animal Production Technology, Faculty of Agricultural Technology, 46000 Thailand, e-mail: nukoon.ka@ksu.ac.th
Aonanong Khundech, Sakon Nakhon Rajabhat University, Fisheries Program, Faculty of Agricultural Technology, 47000 Sakon Nakhon, Thailand; Aquatic Animal Production and Aquaculture Innovation Research Unit, Center of Excellence in Modern Agriculture, Sakon Nakhon Rajabhat University, 47000 Sakon Nakhon, Thailand, e-mail: aonanong.c@snru.ac.th

How to cite this article:
Kanchan C., Imjai P., Chantabut L., Panchai K., Kanchan N., Khundech A., 2023 Synergistic effect of *Piper sarmentosum* and *Terminalia catappa* extracts against *Aeromonas hydrophila* infection in *Rana rugulosa*. AACL Bioflux 16(5):2546-2553.