International Journal of Agricultural Technology 2022Vol. 18(1):49-62
Available online http://www.ijat-aatsea.com
ISSN 2630-0192 (Online)

Antibacterial and anti-biofilm formation activities of high heat tolerant herbal extracts against white feces syndrome-associated *Vibrio parahaemolyticus*

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Bunserm, O., Choonut, A., U-taynapun, K. and Chirapongsatonkul, N. (2022). Antibacterial and anti-biofilm formation activities of high heat tolerant herbal extracts against white feces syndrome-associated *Vibrio parahaemolyticus*. International Journal of Agricultural Technology 18(1):49-62.

Abstract White feces syndrome (WFS), one of *Vibrio parahaemolyticus* outbreaks responsible for 10 to 15% loss in shrimp production, has recently been reported. To reduce the losses from WFS, farmers are forced to use expensive antibiotics or probiotics. Several herbal extracts effectively working out in the prevention and control of WFS have also been recorded. However, heat or improper store may cause a deterioration of the herbal extract efficacy. High heat tolerant herbal (HHTH) extracts were therefore focused in this study. Six ethanolic herbal extracts, originated from *Caesalpinia sappan*, *Pluchea indica*, *Cinnamomum cassia*, *Alpinia galanga*, *Ocimum gratissimum*, and *Origanum vulgare*, were screened for their antibacterial activities by disc diffusion method in the forms of heat-treated (heating at 121^{o}C for 15 min) and nonheated. Two of six, *C. sappan* and *A. galanga* could be served as the potent HHTH extract. The highest inhibitory activity expressing substance, the HHTH extract of *C. sappan*, was selected to further determine for its antibacterial and anti-biofilm activities. The minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) against WFS-associated *V. parahaemolyticus* was the same value of 0.39 mg/ml. Treatment of the test *V. parahaemolyticus* with the HHTH extract of *C. sappan* at MIC showed 79.48±1.11% of the biofilm formation inhibition. Scanning electron microscope (SEM) images revealed that no biofilm formation in *V. parahaemolyticus* treated with the HHTH extract of *C. sappan* compared to the control. This study elucidated that the HHTH extract of *C. sappan* could be served as the potential antibacterial and anti-biofilm agent against WFS-associated *V. parahaemolyticus* which may be used as an alternative natural substance for bacterial control in sustainable aquaculture.

Keywords: *Caesalpinia sappan*, High heat tolerant herbal extract, *Vibrio parahaemolyticus*, White feces syndrome, White shrimp

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Introduction

Pacific white shrimp (*Litopenaeus vannamei*) is one of the world's primadonna aquaculture commodities with high economic value and a large export market (Kurniawinata *et al*., 2021). However, the risk of disease outbreaks is increased in commercial aquaculture productions. Among several factors contributing to disease outbreaks, bacterial infections have been recognized as a major impediment to aquaculture production. Bacteria are major group of pathogens in aquaculture system, generating serious damage during culture and lead to production losses (Delphino *et al*., 2019; Huang *et al*., 2019). White feces syndrome (WFS), one of *Vibrio parahaemolyticus* outbreaks responsible for 10 to 15% loss in shrimp production, has recently been reported. The growth of *V. parahaemolyticus* is initially controlled by the use of antibiotics. This practice increases the risks of antibiotic residues in shrimp tissues and shrimp products. Nowadays, researchers have reported that *Vibrio* species exhibit relatively high antimicrobial resistance, resulting in promoting difficulty to treat infections (Jeyasanta *et al*., 2017). In addition, wide use of antibiotics to treat bacterial infections in aquaculture leads to increasing the number of resistant bacteria (Vivekanandhan *et al*., 2002).

Application of herbal extracts showing bacterial control property is an alternative way to avoid using antibiotic and decrease problems of antibiotic resistant bacteria (Citarasu *et al*., 2010). Previous studies have shown that herbs containing flavonoids and anthocyanin has antimicrobial properties (Khalaphallah and Soliman, 2014; Jantrapanukorn *et al*., 2017). The natural substances exhibiting not only the effective control the growth of bacterial pathogens but also the potential to anti-bofilm formation is increasing concerened. Since biofilm has been remarkably noticed for its effect on the increased resistance of microorganisms to negative environmental conditions such as antibiotics and antimicrobial agents. Therefore, the discoveries of new antibacterial agents and natural products that can regulate bioflm formation and development have recently focused (Lu *et al*., 2019). However, heat or improper storage may cause a deterioration of the herbal extract efficacy. Therefore, there has been an increasing interest in exploring the herbal extracts that contain a constant antibacterial activity after long-time storage at medium-to-high temperature or define as heat tolerance.

In this paper, the antibacterial and anti-biofilm formation activities of the high heat tolerant herbal (HHTH) extract against the WFS-associated *V. parahaemolyticus* was focused. Six herbal extracts, which have been previously demonstrated for its antibacterial activity against diverse pathogenic bacteria, was tested with the representative *V. parahaemolyticus* isolate. The inhibitory

activity of each extract was compared between the heat-treated and nonheated forms through paper disc diffusion method. The potent HHTH extract was further evaluated for the values of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The biofilm formation was measured by crystal violet assay and scanning electron microscope (SEM). The obtained results could provide the effective high heat tolerant natural active compound containing antibacterial properties which can be developed to an alternative substance to replace antibiotics applied in aquaculture.

Materials and methods

Herbal materials

Six herbs including *Caesalpinia sappan* (woods), *Pluchea indica* (leaves), *Cinnamomum cassia* (barks), *Alpinia galanga* (rhizomess), *Ocimum gratissimum* (leaves), and *Origanum vulgare* (leaves), were purchased from local market in Nakhon Si Thammarat Province, Thailand. These herbal samples were cleaned and air-dried before drying in a hot air oven at 45 ℃ for 48 h. The dried samples were then ground into a fine powder using an electric blender. The obtained powder was stored in airtight containers and kept at room temperature until used (El-Mahmood and Doughari, 2008).

Preparation of the ethanol extracts of herbal materials

One hundred grams of the herbal powders were suspended in 95% (v/v) ethanol at a ratio of 1:10. After 72 h, the liquid solution was separated and dried using a rotary evaporator (BUCHI, Operation Manual Rotavapor® R-3) to remove the solvent. Thereafter, the extract was divided into 2 parts; nonheated and heated by autoclave of 121 ℃ for 15 min and named as high heat tolerant herbal extracts (HHTH extracts). The obtained crude extracts were weighed and the yields of each herbal extract were then calculated.

Bacterial strain and growth condition

V. parahaemolyticus used in this study was isolated from the shrimps expressing WSF collected from grow-out ponds in Nakhon Si Thammarat, Thailand. The strain was kept in a house culture collection (Department of Agricultural Science, Faculty of Agriculture, Rajamangala University of Technology Srivijaya, Nakhon Si Thammarat, Thailand). Bacteria in 20% (v/v) glycerol were stored in a refrigerator at -80 ℃ and recultured every one month.

The *V. parahaemolyticus* cells were inoculated in tryptic soy broth (TSB, Difco) containing 1.5% (w/v) NaCl (TSB^+), and cultured with shaking (150 rpm) at 37℃ for 18-24 h. The bacterial cells were then centrifuged 8,000 x g for 10 min, resuspended in sterile saline solution, and then adjusted to a desired concentration (10^7 CFU/ml, OD_{600} of approximately 0.5) (Chaweepack *et al*., 2015).

Antibacterial activity of HHTH extracts

Screening for the antibacterial activity of the nonheated and HHTH extracts of six herbs to inhibit the growth of WSF-associated *V. parahaemolyticus* was determined following the paper disc diffusion method of adapted from Oonmetta-aree *et al*. (2006). The test nonheated and HHTH extracts were prepared by dissolving in 30% (v/v) ethanol to an initial concentration of 200 mg/ml then filtered through a 0.45 µm membrane filter (Whatman). Sterile paper discs were impregnated with 30 µl of either filtered nonheated or HHTH extracts while 30% (v/v) ethanol was used as a control, then dried under aseptic condition. The inoculums of *V. parahaemolyticus* (10^7 CFU/ml) were inoculated onto the Mueller Hinton agar (MHA, HiMeadia) supplemented with 1.5% (w/v) NaCl (MHA^+). Thereafter, the paper discs containing the test solutions were then placed onto the *V. parahaemolyticus*-MHA^+ plate, followed by incubation at 37℃ for 18-24 h. The efficacy of the herbal extracts for the growth inhibition of *V. parahaemolyticus* was estimated by measuring the diameter of the clear zone surrounding the test disc. The HHTH extract showing the highest inhibition zone was selected as a potent substance for further determination of MIC, MBC and anti-biofilm activity.

Determination of MIC and MBC values of the potent HHTH extract

MIC was determined using the resazurin-based turbidometric (TB) assay following a slight modification of Teh *et al*. (2017). In a 96-well flat-bottom plate, the assay composed of 2 vertical rows of the HHTH extract sterility control), 2 vertical rows of the HHTH test sample, 2 vertical rows of mock control which contained an equal ethanol concentration as in the HHTH, 2 vertcal rows of bacterial growth control, and 2 vertcal rows of antibiotic (ceftriaxone) control. All eight wells, in each vertical row, were filled with 100 µl of MHB^+ (Mueller Hinton broth (Himedia) containing 1.5% (w/v) NaCl). The first well of each vertical row contained 100 µl of the sterile HHTH, resuspension solution, sterline saline solution, and ceftriaxone, for the HHTH sterility and HHTH test, mock control, bacterial growth, and antibiotic control,

respectively. The mixture in the first well of all rows (except the antibiotic control) was thoroughly mixed and then subsequently transferred 100 µl into the second well to make a 2-fold dilution. Then the serial dilution was continued from the second to eighth well, and 100 µl was discarded from the eighth well. The final concentration of the HHTH and mock control was one-half of the original concentration in each well except that of the antibiotic control of which the concentration was equal in all wells. Then, 50 µl of the bacterial suspension was put into each well while the sterile saline solution was replaced in the HHTH sterility control column. Microdilution was performed in triplicate. After overnignt incubation at 37℃, 50 µl of 0.1% of resazurin indicator solution was added to all wells and incubated at 37℃ for another 30 min. Changes of solution color was observed and recorded. The lowest concentration prior to color change (from blue to pink) was recorded as the MIC.

To determine the MBC, 5 µl of suspension from all the wells that did not show any change in color were inoculated on TSB^+ and incubated at 37℃. After incubation for 12 h, the lowest concentration with no visible growth was considered as the MBC.

Examination of biofilm formation and scanning electron microscopy (SEM)

Biofilm formation was analyzed by crystal violet assay following the method described by Lu *et al*. (2021) with some modification. Fifty µl of sterile TSB^+ was added into the 96-well microtiter plate, then 50 µl of the potent HHTH extract, at the MIC concentration and double diluted from 1/4, 1/2, and MIC was added into the first column well. After mixed thoroughly, 50 µl of *V. parahaemolyticus* suspensions were added at an initial concentration of 10^7 CFU/ml. The sterile saline was used as the control. After incubated at 37℃ for 24 h, the planktonic cells were washed three times with 250 µl saline, and then dehydrated for 15 min. The biofilms were stained with 0.1% (w/v) crystal violet for 10 min, rinsed three times with 250 µl saline. The dye was dissolved for 10 min with 95% (v/v) ethanol and the OD was measured at 570 nm.

The qualitative assay for biofilm formation was conducted by observation through SEM images with the slight modification of the method of Guo *et al*. (2019). *V. parahaemolyticus* were cultured in TSB^+ at 37^{o}C for 18-24 h, adjusted to OD_{600} of 0.5, then transferred into the culture media containing the potent HHTH extract at the concentrations of 1/4, 1/2 and 1 of MIC. These suspensions were incubated at 37℃ for 24 h. Twenty µl of each suspension was applied to nuclear pore polycarbonate membranes, and fixed in 2.5% glutaraldehyde at 4^{o}C for 24 h. Bacterial cells dehydration process was

achieved by increasing concentrations of ethanol (from 30%, 50%, 70%, 80%, up to 100%). The dried samples were mounted onto stubs, coated with 40-60 nm of gold and then observed by SEM (Zeiss/Merlin compact).

Statistical analysis

One-way analysis of variance (ANOVA) was performed via the SPSS Statistics software version 16.0 (SPSS Inc.). The significant differences among data sets were analyzed using a multiple comparison by Duncan's Multiple Range Test (DMRT). Significant differences were stated at $P<0.05$.

Results

The obtained herbal extracts and its efficacy on antibacterial activity of V. parahaemolyticus

The obtained extracts from 6 selected herbs were characterized and calculated for its percentage yield as shown in Table 1. The highest yield was obtained from *C. cassia* (23.51%) followed by *O. vulgare* (13.69%), *O. gratissimum* (12.80%), *P. indica* (11.31%), *A. galanga* (7.05%) and *C. sappan* (3.97%), respectively.

Table 1. The percentage yield of ethanolic extract of the herbal plants

Herbal plant	Color of extract	Extraction yield (%)
Caesalpinia sappan	Walnut brown	3.97
Pluchea indica	Greenish black	11.31
Cinnamomum cassia	Reddish black	23.51
Alpinia galanga	Walnut brown	7.05
Ocimum gratissimum	Greenish black	12.80
Origanum vulgare	Greenish black	13.69

The results of antibacterial activity of 6 herbal extracts against the WSF-associated *V. parahaemolyticus* are shown in Table 2. The extracts of *C. sappan* and *A. galanga*, both nonheated and HHTH extract, were able to inhibit the growth of *V. parahaemolyticus*. In nonheated form, the crude extract of *C. sappan* had highest inhibitory activity (11.67 ± 0.29 mm), followed by the extract of *A. galanga* (9.50 ± 0.22 mm). Similarly, the HHTH of *C. sappan* showed the largest inhibition zone (13.17 ± 0.13 mm), which was significantly higher than that of *A. galanga* (10.71 ± 0.31 mm). Therefore, the HHTH extract of *C. sappan* was selected as the potent HHTH extract for further determination

for more detail in its antibacterial (MIC and MBC values) and anti-biofilm activities.

Table 2. Antimicrobial activity of the herbal extracts

Bacterium	Herbal extracts	Zone of inhibition (mm)[1]	
		Non-heated	High heat treatment
V. parahaemolyticus	*C. sappan*	11.67 ±0.29[a]	13.17 ±0.13[a]
	P. indica	ND	ND
	C. cassia	ND	ND
	A. galanga	9.50 ±0.22[b]	10.71 ±0.31[b]
	O. gratissimum	ND	ND
	O. vulgare	ND	ND

[1/] Determined by disc diffusion method. The results express as mean ±SD while ND means not detect. Values in the same column with different letters are signifcantly different ($P<0.05$).

Determination of MIC and MBC values of the potent HHTH extract of C. sappan

MIC of the HHTH extract of *C. sappan* was determined using resazurin as an indicator of *V. parahaemolyticus* viability in 96-well microplates. All wells in the growth control as well as mock control, which had ethanol at equal concentration as in the test HHTH extract (containing growth medium, sterile saline solution and bacteria) had changed from blue to pink (Figure 1A, in panels of *Vp* and Alc & *Vp*, respectively. As expected, blue color referred to no bacterial growth was observed in the antibiotic control wells (Figure 1A). Since the HHTH extract of *C. sappan* had a background color of walnut brown, the changed color indicted bacterial growth could be compared with those of the HHTH extract sterility control (no bacteria). The result showed that MIC of *C. sappan* against *V. parahaemolyticus* was considerably recorded at 0.39 mg/ml (Figure 1A).

The MBC value was evaluated by subculturing the HHTH extract of *C. sappan* with no visible growth in the MIC assay (no color change occurred). The pink solution from mock control (Alc & *Vp*) and bacterial growth control (*Vp*) were comparatively subcultured. We found that the MBC of the HHTH extract of *C. sappan* was the same value of MIC at 0.39 mg/ml (Figure 1B). The MIC and MBC values of the potent HHTH extract of *C. sappan* against the test *V. parahaemolyticus* are shown in Table 3.

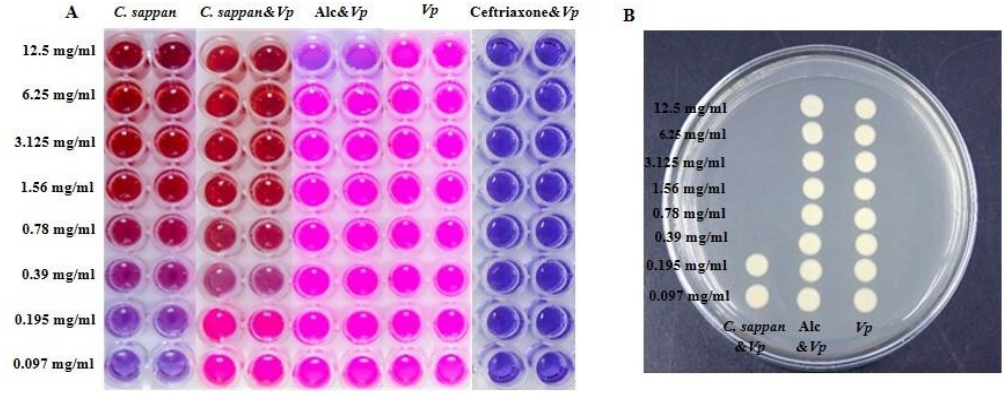

Figure 1. Anti-*V. parahaemolyticus* activity of the HHTH extract of *C. sappan*; **(A)** MIC determination through resazurin-based assay and **(B)** MBC assay

Table 3. The MIC and MBC of the HHTH extract of *C. sappan*

Bacterium	HHTH extract of *C. sappan*	
	MIC	MBC
V. parahaemolyticus	0.39 mg/ml	0.39 mg/ml

Examination of biofilm formation

Detection of biofilm formation by crystal violet assay

The result of biofilm formation analyzed by crystal violet assay suggested that the inhibition ability of the HHTH extract of *C. sappan* increased in a dose-dependent manner. The %inhibition of biofilm produced by *V. parahaemolyticus* was approximately 40%, 50%, and 80% in the treatment 1/4MIC, 1/2MIC and MIC, respectively (Figure 2A). At the MIC of the HHTH extract of *C. sappan*, *%*inhibition was significantly higher than those observed in 1/2MIC and 1/4MIC. The biofilm forming visualized by crystal violet staining in Figure 2B was comparable with the %inhibition shown in Figure 2A.

Biofilm formation inhibition measurement by SEM

SEM was used to assess the anti-biofilm potential of the HTHH extract of *C. sappan* by observation of biofilm morphological structures (Figure 3). The untreated *V. parahaemolyticus* showed a dense biofilm layer and extracellular matrix on the surface of the glass slides after incubation for 24 h (Figure 3A). By contrast, *V. parahaemolyticus* treated with the HTHH extract of *C. sappan* at the test concenrations of 1/4MIC, 1/2MIC, and MIC for 24 h. showed lower

biofilm content in comparison with the control. Moreover, individual cells were loosely attached to the surface (Figure 3B-D).

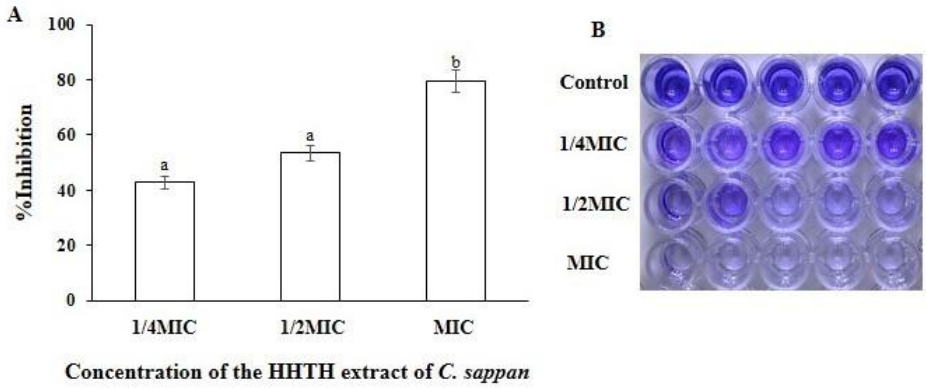

Figure 2. Biofilm formation assay in *V. parahaemolyticus* treated with different concentration of the HHTH extract of *C. sappan*, **(A)** Quantification of crystal violet dye attached to the biofilm forming cells. Bars represent the standard deviation from triplicate determinations and **(B)** Crystal violet stained plate of *V. parahaemolyticus*

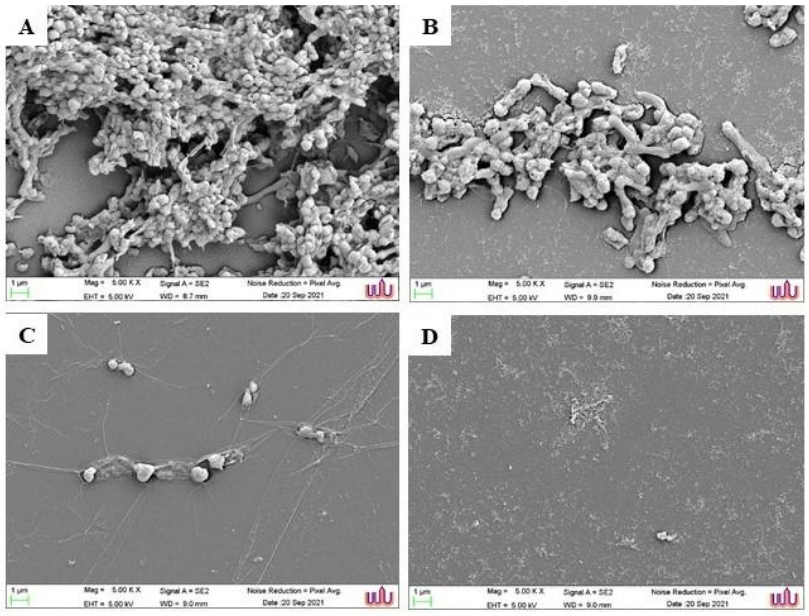

Figure 3. Scanning electron microscopy (SEM) images of biofilm formation of *V. parahaemolyticus* treated with the HHTH extract of *C. sappan*; **(A)** Control (no HHTH extract), **(B)** 1/4MIC, **(C)** 1/2MIC, and **(D)** MIC for 24 h. SEM pictures were taken at 5,000X magnification

Discussion

Among the known pathogenic bacteria, *Vibrio* spp., particularly *V. parahaemolyticus*, contributes to a high mortality which considerably associated to WFS in shrimp culture causing economic losses (Tang *et al*., 2016). In order to reduce the losses, farmers have used antibiotics to overcome these bacterial pathogens. The adverse effect of antibiotic-resistant bacteria existence has raisen consequently. The alternative natural substances including effective herbal extracts have been introduced in the prevention and control of *Vibrio* spp. There have been noticed that most natural substances have a limited shelf-life. In addition, heat or improper storage may cause a deterioration of the herbal extract efficacy. This maybe due to possibility of losing their bioactivity through the decomposition of active phytochemicals after heat treatment (Negi, 2012).

In this research, 6 herbal plant materials were extracted using ethanol as the solvent and the obtained crude extracts were tested for their antibacterial activity against WSF-associated *V. parahaemolyticus.* The inhibitory efficiency was comparatively evaluated between the extracts in the forms of nonheated and high heat treated (HHTH extract). We found that the ethanolic extracts of *C. sappan* and *A. galanga* were able to inhibit the growth of *V. parahaemolyticus* and could be considered as the effective HHTH extracts. Other ethanolic extracts from the leaves of *P. indica*, *O. gratissimum*, and *O. vulgare*, and the inner barks of *C. cassia* could not inhibit *V. parahaemolyticus*. These findings were inconsistent with the previous researches (Igbinosa and Idemudia, 2016; Zheng *et al*., 2020; Das *et al*., 2021). This can lead to speculation that natural bioactive compounds may give different effects on diverse bacterial species or strains. Surprisingly, our results showed that the inhibitory efficacy of the HHTH extracts of *C. sappan* and *A. galanga* was greater than the nonheated extract. Our findings revealed that constituents in the HHTH extracts of *C. sappan* and *A. galanga* responsible for their antibacterial activity were thermo-stable compounds. The thermal stability of herbal extract is also reported in the methanolic extracts of *Hypericum alpestre* and *Sanguisorba officinalis* treated at 121^{o}C for either 30 or 60 min for their antibacterial activity against *Staphylococcus aureus* MDC 5233 strain. On the other hand, the antibacterial activity has been lost in the extracts of *Agrimonia eupatoria* and *Rumex obtusifolius* (Ginovyan, 2017). Brazilin and essential oils containing 1,8-cineole, 4-allylphenyl acetate, and methyl eugenol, have been reported as active compounds, which exhibit strong antibacterial activity, in *C. sappan* and *A. galanga* (Xu *et al*., 2004; Bakkali *et al*., 2008: Nirmal and Panichayupakaranant, 2015). However, the thermo-stability of these compounds has not been reported.

Since the HHTH extract of *C. sappan* exhibited the highest inhibitory effect on the growth of *V. parahaemolyticus*, it was selected for further study in more detail of antibacterial and anti-biofilm formation. The obtained results showed that MIC and MBC of the HHTH extract of *C. sappan* were the same value of 0.39 mg/ml. Then the capacity to reduce the biofilm formation was determined at the MIC and its 2-fold dilutions of 1/2MIC and 1/4MIC. Biofilm reffers to a complex microbial community characterized by livings attached to a surface or interface which are enclosed in an exopolysaccharide matrix (Eps) of microbial and host (Costerton *et al*., 1995). Accumulated evidences have demonstrated that the biofilm formation results in enhancement of bacterial resistance to negative environmental influences. Bacteria living in the formed biofilm are found to have a highly elevated pattern of adaptive resistance to antibiotics and other disinfectants compared to planktonic compartments (Lu *et al*., 2019) which may involve in their increased virulence (Overhage *et al*., 2008). The biofilm elimination was found to be positively correlated with the concentrations of the treated HHTH extract. In addition, the morphological structures of the biofilm were obviously differed among the test concentrations. These findings suggested that there were high heat torelant constituents in our HHTH extract of *C. sappan*. The effect on reducing biofilm formation may possibly relys on (1) antibacterial activity to kill or reduce number of bacterial cells, (2) disposal the biofilm production mechanism (inhibition of the adhesive matrix and microbial attachment production) and (3) interruption a cell-to-cell communication, quorum sensing (QS), signaling (Lu *et al*., 2019). Several literatures have reported that natural products from plants have antimicrobial and anti-bioflm formation ability. For example, Lu *et al*. (2021) have shown that a MIC of cinnamon extract was effective in preventing biofilm formation of *V. parahaemolyticus* while Santhakumari *et al*. (2018) have demonstrated the anti-biofilm activity of 2,6-di-tert-butyl-4-methylphenol on *V. parahaemolyticus.* Persson *et al.* (2005) have reported N-(heptylsulfanylacetyl)-l-homoserine lactone is a potent QS inhibitor in garlic extract that possesses the inhibitory effects on biofilm formation.

To our knowledge, this is the first report that has assessed the plausibility efficacy of the HHTH extract of *C. sappan* on the antibacterial and anti-biofilm activities against WSF-associated *V. parahaemolyticus.* However, the mechanisms underlying antibacterial and anti-biofilm formation as well as the bioactive compounds in the HHTH extract of *C. sappan* could be further investigated. This newly discovered high heat tolerant natural antibacterial and anti-biofilm agent is a promising candidate for application in the control of pathogenic *Vibrio* spp. to reduce losses in aquaculture.

Acknowledgements

This research was a part of the project entitled "Development of heat-stable Thai herbal extract product inhibiting *Vibrio* spp. causing white feces syndrome in white shrimp (*Litopenaeus vannamei*)" financially supported by Public and Private Linkage for Commercialize Research Project, Office of the Permanent Secretary (OPS), Ministry of Higher Education, Science, Research and Innovation (MHESI), Thailand and Yeast Master Co., Ltd. Finally, we would like to express gratitude to all partners for supporting.

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(Received: 21 August 2021, accepted: 30 October 2021)