Asploro Journal of Biomedical and Clinical Case Reports
(ISSN: 2582-0370)

Original Article

DOI: https://doi.org/10.36502/2019/ASJBCCR.6173

Antibacterial Activity of Extracts from Dried and Fresh Herbal Plant (*Phyllanthus amarus*) Against Pathogens Causing Acute Hepatopancreatic Necrosis Disease (Ahpnd) in White Leg Shrimp (*Litopenaeus vannamei*) at Thua Thien Hue Province, Vietnam

Phuong TV[1], Hai Yen PT[2], Linh NQ[1,3*]

[1]Institute of Biotechnology, Hue University, Road No.10, Phu Vang, Thua Thien Hue, 530000, Vietnam
[2]Faculty of Fisheries, University of Agriculture and Forestry, Hue University, 102 Phung Hung St., Hue, 530000, Vietnam
[3]Hue University, 3 Le Loi St., Hue, 530000, Vietnam

Corresponding Author: **Nguyen Quang Linh**
Address: Institute of Biotechnology, Hue University, Road No.10, Phu Vang, Thua Thien Hue, 530000, Vietnam; E-mail: nguyenquanglinh@hueuni.edu.vn; phamthihaiyen@hueuni.edu.vn
Received date: 24 October 2019; **Accepted date**: 16 November 2019; **Published date**: 22 November 2019

Citation: Phuong TV, Hai Yen PT, Linh NQ. Antibacterial Activity of Extracts from Dried and Fresh Herbal Plant (*Phyllanthus amarus*) Against Pathogens Causing Acute Hepatopancreatic Necrosis Disease (Ahpnd) in White Leg Shrimp (*Litopenaeus vannamei*) at Thua Thien Hue Province, Vietnam. Asp Biomed Clin Case Rep. 2019 Nov 22;2(3):120-128.

Abstract

The study aimed to determine extract yield (%), antibacterial activity, and minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of plant extracts from dried and fresh herbal plants (*Phyllanthus amarus*) against *Vibrio parahaemolyticus* strain causing acute hepatopancreatic necrosis disease (AHPND) in white leg shrimp (*L. vannamei*). The result showed that the extract yields of dry and fresh herbs reached 11.50% and 2.75%, respectively and the antibacterial activity of the two extracts both are good at concentrations from 250 to 1,000 mg/mL at the same bacterial density of 10^6 CFU/mL. Specifically, the diameter of the inhibition zone at 250; 500; 750 and 1,000 mg/mL concentration of dried herbal extracts reached 16.75±0.96; 18.50±1.29; 20.75±0.96 and 21.25±0.50 mm, while that of fresh herbal extracts reached 14.50±1.29; 16.25±0.50; 16.75±0.50 and 17.00±0.00 mm, respectively, with a statistically significant difference $p<0.05$. The result also showed that MIC values of dried and fresh extracts were defined at 125 mg/mL and 250 mg/mL, respectively and that MBC values of the extracts were 500 and 1,000 mg/mL respectively. The GC-MS analysis revealed that there were 19 natural compounds in the dried extract, in which Ethyl Linoleolate ($C_{20}H_{36}O_2$) compound occupied the highest ratio (22.43 %), while 2.3-Dihydro-3.5-dihydroxy-6-methy-4H-pyran-4-one ($C_6H_8O_4$) was the lowest (0.24 %).

Keywords

EMS; AHPND; Herbs; Extract; *Phyllanthus amarus*; MIC/MBC

Introduction

Vietnam shrimp farming industry has brought great economic efficiency and has also become one of the key economic sectors of the country. The revenue of the industrial brackish shrimp export was estimated to reach $4.2 billion in 2019 (DOF, 2019). White leg

shrimp (*Litopenaeus vannamei*) is one of species which has big harvest and high economic value [1]. Acute Hepatopancreatic Necrosis Disease (AHPND), also called Early Mortality Syndrome (EMS) in shrimp is caused by *Vibrio bacteria*. This disease can lead to death rate up to 100 % in the habitats of both white leg shrimp (*L. vannamei)* and black tiger shrimp (*Penaeus monodon*), which causes considerable losses to shrimp farming. Clinical signs of the disease include empty gastrointestinal tracts, opaque white stomachs, white atopic livers, lethargic shrimp, anorexia, and soft shells (Leano và Mohan, 2012). AHPND disease was first noticed in China in 2009, and then found in other parts of Asia, such as: Vietnam (2010), Malaysia (2011), Thailand (2012), [2,3] and Mexico (2013) [4-6]. The causative agent of AHPND/EMS was indentified to be *Vibrio parahaemolyticus* strain [7] that harbors a pVA plasmid encoding toxins PirAVp and PirBVp [8]. As also reported by Giang *et al*., (2016), three bacterial strains were determined, including: *V. parahaemolyticus*; *V. alginolyticus* and *V. vulnificus* with highest encountering frequency of more than 60% in disease shrimp samples. In which, the PCR results determined the presence of *V. parahaemolyticus* bacterial which is considered a causative agent of acute hepatopancreatic necrosis disease [9]. Meanwhile, the new analysis results of Jee Eun Han *et al*, (2017) showed that all four *Vibrio* strains (16-902/1, 16-903/1, 16-904/1 and 16-905/1) were isolated from either stomaches of diseased shrimp or sediment samples from AHPND-affected farms in Latin America during 2016. Bacterial identifications were carried out using 16S rRNA sequencing and *Vibrio*-specific PCR assays targeting hly gene. By the PCR assays, these 4 strains were identified to be *V. campbellii* by 16S rRNA sequence analysis and hly gene PCR. These *V. campbellii* strains had both pirAvp and pirBvp genes [10].

According to Linh NQ (2014), *V. parahaemolyticus* V1 strain was isolated from EMS disease juvenile shrimp samples in Thua Thien Hue province with density from 10^3 - 10^4 CFU/mL in the incubation period [11]. According to Citarasu (2010), the herbs have beneficial properties such as growth stimulant, immune booster, antibacterial and anti-fugus). Many other researches also proved that there are many kind of herbs such as: guava leaves (*Psidium guajava*), betel leaves (*Piper betel* L.), *Phyllanthus amarus* and seeds of Myrtle (*Rhodomyrtus tomentosa*), which have antibacterial properties against bacteria causing AHPND in white leg shrimps including *V. parahaemolyticus* KC12.020; *V. parahaemolyticus* KC13.14.2 and *V. harveyi* KC13.17.5 strain [12,13]. Herbs are also used for flavoring, stimulating gastrointestinal secretion, thus increasing feed intake as well as decreasing feed conversation rate (Venketramalingam et al, 2007). Besides, not only this herb is studied for disease prevention and treatment for white-leg shrimp (*L. vannamei*), but many other herbs are also studied for disease prevention and treatment for Indian white shrimp (*Fenneropenaeus indicus*), specifically: the three methanolic antibacterial extracts, *Psoralea corylifolia*, *Murraya koenigii* and *Quercus infectoria* effectively suppressed the shrimp bacterial pathogens isolated from infected *F. indicus* gut. The average zone of inhibition was observed ranging between 9.00 to 14.00 mm against the selected bacterial pathogens including *Pseudomanas aeruginosa*, *Staphylococus aureus* and *V. harveyi* strains [14]. Another research illustrated that using *Phyllanthus amarus* extracted by ethanol to enrich *Artemia nauplii* produced the best SR, WG and SGR (96.6%, 1.01g, and 4.1%, respectively against the control 82%, 0.6g and 3.9%, respectively) in *Macrobrachium rosenbergii* PL [15].

Material and Research Methods

Material Preparation:

1. Herbal Plant: The *P. amarus* (**Fig-1**) was

Fig-1: *Phyllanthus amarus*

collected from January to March 2019 at the mountainous area of Thua Thien Hue province, Vietnam. The chosen herbal plants were mature ones of which average weight reached 3,25 g and average height 28,82 cm. The samples had to be fresh and not crushed, have green colour. They were washed using fresh water.

2. Bacterial Strain: *Vibrio parahaemolyticus* strain identified to carry two PirAVp, PirBVp toxin gene was isolated from disease shrimp samples (AHPND) in farms at Thua Thien Hue, Vietnamand labeled. The samples' average weight was 0.36 g. The experiment was carried out in a sterile laboratory (Labcaire VLF-R) at the Institute of Biotechnology of Hue University. Bacterial strain was kept at -80 ^{o}C and then restored in enrichment culture medium, specifically Tryptic Soy Brorth (TSB) supplemented with 2% NaCl in 2-tray shaking incubator (GFL 3032) at the temperature of 37 ^{o}C, with the shaking frequency of 180 rpm for 24 hours to collect bacterial outbreak. Subsequently, the bacterial density was determined using optical density (OD) measuring method by UV-VIS spectroscopy (U2900, Hitachi, Japan) at 600 nm wavelength. The bacterial density was adjusted to 10^6 CFU/mL (OD = 1, equivalent to a bacterial density of 10^8 CFU/mL) to test antibacterial activity compared to initial bacterial density.
3. Antimicrobial Preparation: Herbal extracts were mixed in sterile distilled water (diluted at 1g/1 mL ratio) into concentrations of 1,000, 750, 500 and 250 mg/mL. Negative control was sterile distilled water.

Herbal Extraction Method:

The powdered herbal (400 g, d=1 mm) and fresh herbal (400 g, pureed) were soaked in 70% ethanol (1:5 ratio of herbal plant:ethanol) and stirred for 48 hours, then filtered through vacuum filtration system (Rocker 300-LF31) on Whatman filter paper No. 4 (code: 1004042, diameter of 20-25 µm). After the primary herbal extract was separated, the remaining were soaked in 40% ethanol (1:5 ratio) for 48 hours, and another batch of herbal extract was collected. Two batches of extracts were mixed to obtain total extract. They were then taken into rotation at 60 ^{o}C by Heidolph vacuum evaporation system (Germany). The filtrates were evaporated and dried at 50 ^{o}C until there was no more change in weight. The extract yields were stored at 4 ^{o}C and their yield percentages were calculated using the following formula (Turker et al, 2009):

Extracts yield (%) = [Weight of extracted (g)/ weight of raw plant sample (g)] x 100 [16].

Antibacterial Activities of the Herbal Extract:

The bactericidal activity of the extracts was tested by disk diffuse test, also known as Kirby-Bauer method. Testing was done in sterile Labcaire VLF-R. The suspension containing bacterial strain (100 µL, 10^6 CFU/mL) was evenly distributed on agar plates containing a solid alkaline peptone medium. The sterile paper disc (d = 0.6 mm) was put on agar plates surface (including 4 samples and 1 negative control (sterile distilled water). 50 µL of plant extracts with 1,000; 750; 500 and 250 mg/mL concentration was put on paper discs. After that they were stored at 4^{o}C for 8 hours for extracts to spread over the surface of the paper discs with 4 replications. The diameter of inhibition zone was measured after 24; 48; 72 and 96 hours.

Determination of Minimum Inhibitory Concentration of Plant Extract:

Minimum Inhibitory Concentration (MIC) was

Fig-2: *Resazurin*

defined using Satyajit method [17]. 100 µL of bacterial solution was added into each well of a 96-well plate containing 100 µL of the plant extract that was diluted to different concentrations with an initial stock extract of 1,000 mg/mL from 1, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256 concentration. Control wells contain 100 µL of sterile distilled water which was used to dissolve plant extracts. The plates was then incubated at 37 °C for 24 hours, after that each well was added 20 µL resazurin 0,1% (**Fig-2**). MIC value was defined as the lowest concentration of plant extracts (no change resazulin color) that inhibits the bacterial growth.

Determination of Minimum Bactericidal Concentration of Plant Extract:

All test solution from wells with no discoloration of 0.01% resazurin, which is spread on plates of solid alkaline agar medium was absorbed and incubated at 37 °C. Bacterial survival after 24 hours was observed. Minimum Bactericidal Concentration (MBC) was defined as the lowest concentration of plant extract that did not exhibit any bacterial growth on the agar plates (no colonies appeared on the agar plate), while the control plate had bacteria colonies [17].

Analysis of Natural Compounds of Extracts:

Based on the results of the antibacterial test of the above experiments, we selected the extract that gave the optimal results which had the best antibacterial activity. The presence of natural compounds of extract from herbal powder was analyzed using Gas Chromatography Mass Spectrometry.

Statistical Analysis:

Data were expressed as mean ± standard error (mean ± SEM). One-way ANOVA was followed by SPSS 16.0 software. LSD test was used to compare the diameter of inhibitory zones induced by different extract concentrations. In all analysis, significance was established when probability level was ≤ 0.05.

Results and Discussion

Extract yield of the herbal tree (P. amarus):

Extracts from the herbal plant (*P. amarus*) was obtained using vacuum filtration system. After complete ethanol solvent removal, extracts yield was identified as the percentage of the weight of extracted residue and weight of initial raw plant. The results of dried and fresh herbal extraction yield (*P. amarus*) were presented in **Table-1**.

Table-1: Extraction yield of the herbal plant (P. amarus)

Material	Weight (g)	Weight of extract (g)	Yield (%)
Dried	400	46	11.50[a]
Fresh	400	11	2.75[b]

The average weight of extract obtained out of 400 g fresh material was 11.0 g, equivalent extract yield was 2.75 %. Meanwhile, the average weight of extract obtained out of 400 g dried material (dried from 2 kg of fresh herbal plants) was 46.0 g, equivalent extract yield was 11.50 % ($p<0.05$). The research result showed that the dried herbal plant (*P. amarus*) had higher extraction yield than fresh one. According to the research result of Hong Mong Huyen et al., (2018), extraction yield of dried basket plant (*Callisia fragrans* or *Golden Comb* in Vietnam*) was only 10.8 %, while* extraction yield of dried Moringa *(Moringa oleifera*) was 15.0 % [18]. The research by Ashraf A. Mostafa indicated that the highest extraction yield of the plants under experiment belonged to dried powder of *Punica granatum* which was 4.87 g, equivalent to 9.74 % extraction yield [19].

Antibacterial Activity of Dried and Fresh Extracts Against V. parahaemolyticus:

As can be seen in **Table-2**, **Fig-4** and **Fig-5**, all extract concentrations from lowest to highest (250 – 1,000 mg/L) of dried extracts exhibited higher diameters of inhibition zone than those of fresh extracts at bacterial density of 10^6 CFU/mL, ($p<0.05$); specifically diameters of inhibition zone of dried extracts at the concentration of 250; 500; 750 and 1,000 mg/mL were 16.75 ± 0.96, 18.50 ± 1.29, 20.75 ± 0.96, 21.25 ± 0.50 mm, respectively (**Fig-3**), higher than the diameters of inhibition zone of fresh extracts 14.50 ± 1.29, 16.25 ± 0.50, 16.75 ± 0.50 and 17.00 ± 0.00 mm, respectively (**Fig-4**). The difference were statistically significant ($p<0.05$). Of which, at extract concentration of 1,000 mg/mL, highest antibacterial activity against *V. parahaemoliticus* was observed, both in dried (21.25 ± 0.50 mm) and fresh extracts (17.00 ± 0.00). The research by Lua Thi Dang *et al*,

(2018) revealed that diameters of inhibition zone of *P. amarus* at the concentration of 1,000 (µg/plate) against the following bacteria: *V. parahaemolyticus* KC12.020, *V. parahaemolyticus* KC13.14.2, and *V. harveyi* KC13.17.5 were 12.0 ± 1.0, 13.3 ± 1.5 and 13.7 ± 0.6 mm, respectively [13]. In addition, the study of Hong Mong Huyen et al. (2018) illustrated that among the tested 7 herbal plants, antibacterial activity of *Ricinus communis* L at the concentration of 40 mg had the largest antibacterial zone against 2 types of bacteria *V. harveyi* and *V. parahaemolyticus* causing AHPND on white-leg shrimp (*L. vannamei*) antibacterial zone 18,0 ± 1,4 và 17,5 ± 0,70 mm [18], respectively.

Identification of Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) of Dried and Fresh Extract:

Minimum inhibitory concentration MIC is the lowest level of concentration in the series of tested concentration of the extracts that exhibited growth inhibitory of bacteria did not change the colour of resazurin (**Fig-5A** and **5B**). Therefore, the lower minimum inhibitory concentration was, the more

Table-2: Diameters of the inhibition zone of the two types of extracts

Extract concentration (mg/mL)	Bacteria density	Diameter of the inhibition zone (mm) (X±δ)	
		Dried	Fresh
1,000	10^6 CFU/mL	21.25^b ± 0.50	17.00^a ± 0.00
750		20.75^b ± 0.96	16.75^a ± 0.50
500		18.50^b ± 1.29	16.25^a ± 0.50
250		16.75^b ± 0.96	14.50^a ± 1.29

Note: Same letters a b in the same row do not denote statistically significant difference, (p>0.05)

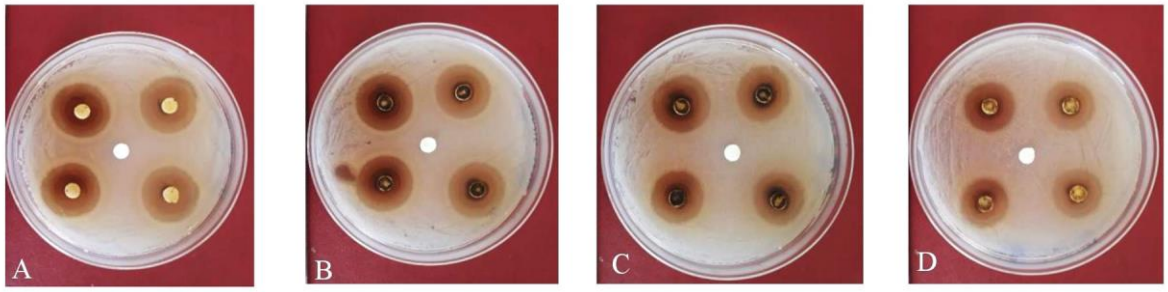


Fig-3:

Vibrio parahaemolyticus resistance of dried P. amarus, (A) 1,000 mg/mL, (B) 750 mg/mL, (C) 500 mg/mL, (D) 250 mg/L

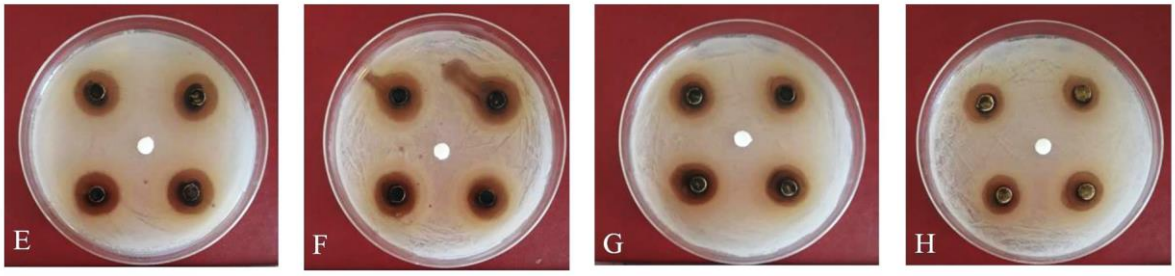


Fig-4:

Vibrio parahaemolyticus resistance of fresh P. amarus, (E) 1,000 mg/mL, (F) 750 mg/mL, (G) 500 mg/mL, (H) 250 mg/L

affective antibacterial activity was. Minimum bactericidal concentration MBC is the lowest level of concentration in the series of tested concentration of the extracts that could eliminate all bacteria in the wells, there appeared no colonies in the agar environment on the plates. Analysis result of MIC and MBC of the dried and fresh herbal plant (*P. amarus*) was presented in **Table-3**.

As can be seen in **Table-3**, extracts of dried herbal plants (*P. amarus*) exhibited MIC at 125 mg/mL, which was lower than that of fresh herbal plants at 250 mg/mL, equivalent ratio of MBC/MIC of both dried and fresh extracts was 4, however MBC of dried extract was 500 mg/mL while MBC of fresh extract was as high as 1.000 mg/mL. MBC of dried extract was optimal than that of fresh extract. According to the report of Canillac and Mourey (2001), if the ratio of MBC/MIC is lower than or equal to 4, the extract is considered bactericidal; in contrast, if this ratio is higher than 4, the extract is bacteriostatic [20]. Therefore, based on the result in **Table-3**., it can be concluded that extract from the herbal plant (*P. amarus*) in both dried and fresh conditions exhibited bactericidal activity against *V. parahaemolyticus* (AHPND) causing disease on white-leg shrimps. Similarly, in the research of Lua Thi Dang *et al*, (2018), extract from the herbal plant (*P. amarus*) exhibited MIC at 312 mg/mL and MBC at 625 mg/mL against 2 strains of bacteria *V. parahaemolyticus* and *V. harveyi* [13]. Meanwhile, extract of *Ricinus communis* L has bactericidal activity against *V. harveyi* và *V. parahaemolyticus* causing disease on shrimps (MBC/MIC = 2) [18].

Natural compounds in the extract from the herbal plant (P. amarus):

After antibacterial activity of extract from dried herbal plant (*P. amarus*) proved superior to fresh one, presence of natural compounds in dried herbal plant (*P. amarus*) was analyzed. The result of compound analysis in 1 g of dried extract was presented in **Fig-6**.

As can be seen in **Fig-6**, there were 19 natural bioactive components in 1g of extract: of which the compound Ethyl Linoleolate ($C_{20}H_{36}O_2$) occupied the highest percent at 22,43 %, Methy linalelate ($C_{19}H_{34}O_2$) followed at 11,72 % while 2,3-Dihydro-3,5-

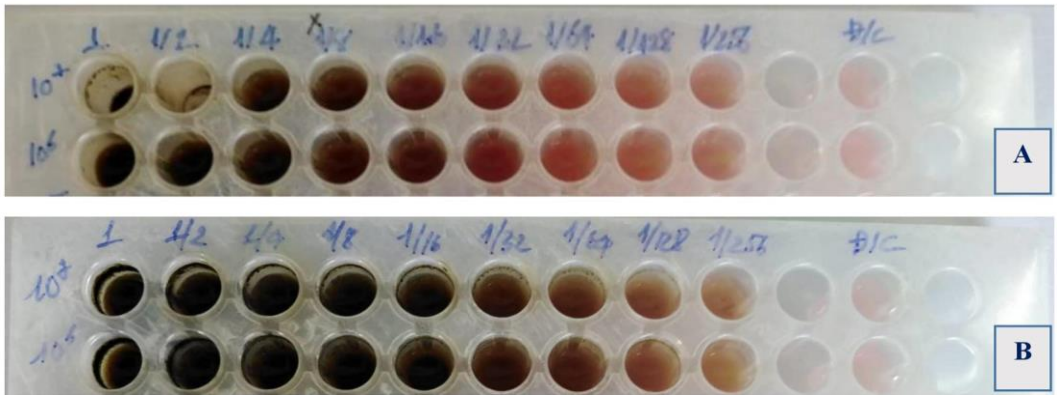
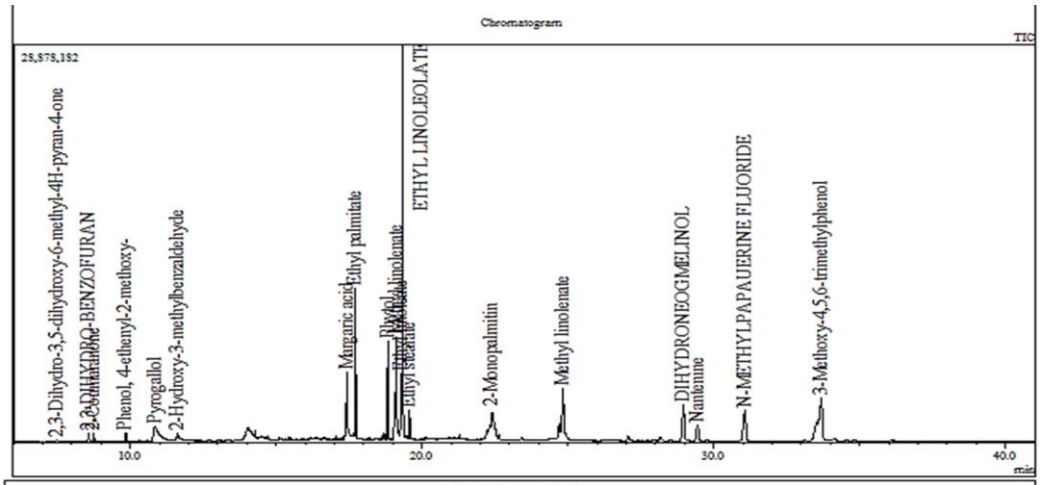


Fig-5A: *Testing by adding resazurin solution onto dried extract*
Fig-5B: *Testing by adding resazurin solution onto fresh extract*

Table-3: MIC and MBC of extracts of dried and fresh herbal plants (*P. amarus*)

Bacterial Cell	Dried extract			Fresh extract		
Density (CFU/mL)	MIC (mg/mL)	MBC (mg/mL)	MBC/MIC	MIC (mg/mL)	MBC (mg/mL)	MBC/MIC
10^6	125	500	4	250	1,000	4

dihydroxy-6-methy-4H-pyran-4-one ($C_6H_8O_4$) only occupied 0,24 %, the lowest percentage among the identified 19 compounds. Arun's Gas Chromatography-Mass Spectrum (GC-MS) Analysis [21] showed that in the extract of *P. amarus* using Acetone there existed 9 bioactive compounds while using ethanol there existed only 7 bioactive compounds which are lower than in our research results.



Peak Report TIC

Peak#	Name	R.Time	Area	Area%
1	2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	7.484	445662	0.24
2	2,3-DIHYDRO-BENZOFURAN	8.569	1218839	0.65
3	2-Coumaranone	8.757	971952	0.52
4	Phenol, 4-ethenyl-2-methoxy-	9.855	1120887	0.60
5	Pyrogallol	10.870	12116959	6.49
6	2-Hydroxy-3-methylbenzaldehyde	11.622	2150119	1.15
7	Margaric acid	17.435	10250519	5.49
8	Ethyl palmitate	17.725	16072970	8.61
9	Phytol	18.830	10836024	5.80
10	Methyl linolenate	19.129	21879860	11.72
11	Ethyl linoleate	19.283	4926747	2.64
12	ETHYL LINOLEOLATE	19.346	41886614	22.43
13	Ethyl stearate	19.580	2954725	1.58
14	2-Monopalmitin	22.409	5119034	2.74
15	Methyl linolenate	24.839	11309492	6.06
16	DIHYDRONEOGMELINOL	28.965	12148035	6.51
17	Nantenine	29.448	5849179	3.13
18	N-METHYLPAPAUERINE FLUORIDE	31.070	12925397	6.92
19	3-Methoxy-4,5,6-trimethylphenol	33.688	12525764	6.71
			186708778	100.00

Fig-6: GC_MS spectrum of dried extract.

Conclusion

Extract from both dried and fresh herbal plant (*P. amarus*) exhibited antibacterial activity against *V. parahaemolyticus* causing AHPND on white-leg shrimp (*L. vannamei*) under experimental conditions. Dried extract has higher extraction yield and antibacterial activity than fresh extract ($p < 0,05$).

Minimum inhibitory concentration (MIC) of extract of dried herbal plant (*P. amarus*) was 125 mg/mL, and of fresh herbal plant was 250 mg/mL, inhibiting bacteria at the concentration of 10^6 CFU/mL.

The ratio of MBC/MIC of both dried and fresh extracts was 4, however MBC of dried extract was 500 mg/mL while MBC of fresh extract was 1.000 mg/mL.

There were 19 natural components in the extracts of the herbal plant (*P. amarus*), of which Ethyl Linoleolate ($C_{20}H_{36}O_2$) occupied the highest percent at 22,43 % while 2,3-Dihydro-3,5-dihydroxy-6-methy-4H-pyran-4-one ($C_6H_8O_4$) occupied the lowest percent at 0,24 %.

Acknowledgements

This article was supported by Hue University and Ministry of Education and Traning project with code number: CT-2018-DHH-07 and students participated in the research.

References

[1] FAO. FAO Yearbook: Fishery and Aquaculture Statistics. http://www.fao.org/3/a-i5716t.pdf. Accessed 18 Dec 2017, 2014.

[2] Joshi J, Srisala J, Truong VH, Chen IT, Nuangsaeng B, Suthienkul O, Lo CF, Flegel TW, Sritunyalucksana K, Thitamadee S. Variation in *Vibrio parahaemolyticus* isolates from a single Thai shrimp farm experiencing an outbreak of acute hepatopancreatic necrosis disease (AHPND). Aquaculture. 2014 May 20;428:297-302.

[3] Tran L, Nunan L, Redman RM, Mohney LL, Pantoja CR, Fitzsimmons K, Lightner DV. Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp. Dis Aquat Organ. 2013 Jul 9;105(1):45-55. [**PMID**: 23836769]

[4] Nunan L, Lightner D, Pantoja C, Gomez-Jimenez S. Detection of acute hepatopancreatic necrosis disease (AHPND) in Mexico. Dis Aquat Organ. 2014 Aug 21;111(1):81-86. [**PMID**: 25144120]

[5] Soto-Rodriguez SA, Gomez-Gil B, Lozano-Olvera R, Betancourt-Lozano M, Morales-Covarrubias MS. Field and experimental evidence of *Vibrio parahaemolyticus* as the causative agent of acute hepatopancreatic necrosis disease of cultured shrimp (*Litopenaeus vannamei*) in Northwestern Mexico. Appl Environ Microbiol. 2015 Mar;81(5):1689-99. [**PMID**: 25548045]

[6] Thitamadee S, Prachumwat A, Srisala J, Jaroenlak P, Salachan PV, Sritunyalucksana K, Flegel TW, Itsathitphaisarn O. Review of current disease threats for cultivated penaeid shrimp in Asia. Aquaculture. 2016 Feb 1;452:69-87.

[7] Quang NN, Linh NQ, Linh NTH, Ha TN. Study on Necrotizing Hepatopancreatic disease in white leg shrimp cultured at Thua Thien Hue province, Vietnam. Journal of Agriculture and Rural development. 2013;17(1). (in Vietnamese)

[8] Phiwsaiya K, Charoensapsri W, Taengphu S, Dong HT, Sangsuriya P, Nguyen GTT, Pham HQ, Amparyup P, Sritunyalucksana K, Taengchaiyaphum S, Chaivisuthangkura P, Longyant S, Sithigorngul P, Senapin S. A Natural *Vibrio parahaemolyticus* ΔpirA ^{Vp} pirB $^{Vp+}$ Mutant Kills Shrimp but Produces neither Pir ^{Vp} Toxins nor Acute Hepatopancreatic Necrosis Disease Lesions. Appl Environ Microbiol. 2017 Aug 1;83(16). pii: e00680-17. [**PMID**: 28576761]

[9] Giang NTT, Toan PV, Hung. Hepotopancreatic Necrosic syndrome in white leg shrimp (*Litopenaeus vannamei*) farmed in Ninh Thuan, Vietnam. Journal of Science and Fisheries Technology. 2015 Mar;31:32-40. (in Vietnamese)

[10] Han JE. Four AHPND strains identified on Latin American shrimp farms. The Global Aquaculture Advocate. 2017.

[11] Dao NTB, Linh NQ, Khanh NV. Research on some characteristics of *Vibrio parahaemolyticus* which causes EMS disease on juvenile shrimp at Dien Huong community, Phong Dien district, Thua Thien Hue, Vietnam. Hue University of journal of Science. 2014 Dec 12;13-22.

[12] Lua DT, Ha LTN, Hai NT. Antibacterial activity of leaf and seed myrtle (*Rhodomyrtus tomentosa*) against Bacterial causing hepatopancreatic necrosis disease in brackish water shrimp. Journal of Science anf Development. 2015 Oct 12;13(7):1101-8. (in Vietnamese)

[13] Nguyen HT, Dang L, Nguyen HT, Hoang H, Lai HT, Nguyen HT. Screening antibacterial effects of Vietnamese plant extracts against pathogens caused acute hepatopancreatic necrosis disease in shrimps. Asian journal of pharmaceutical and Clinical Research. 2018;11(5):77-83.

[14] Velmurugan SU, Citarasu T. Effect of herbal antibacterial extracts on the gut floral changes in Indian white shrimp *Fenneropenaeus indicus*. Romanian Biotechnological Letters. 2010 Nov 1;15(6):5710-17.

[15] Kalaiselvi VC, Saravana Bhavan SP, Kalpana R, Rajkumar G, Satgurunathan T. *Phyllanthus amarus* enriched Artemia nauplii enhanced survival, growth and nutritional quality of early post-larvae of the prawn *Macrobrachium rosenbergii*. Clinical Nutrition and Metabolism. 2018;1(2):1-15.

[16] Turker H, Yıldırım AB, Karakaş FP. Sensitivity of bacteria isolated from fish to some medicinal plants. Turkish Journal of Fisheries and Aquatic Sciences. 2009 Feb 1;9(2):181-86.

[17] Sarker SD, Nahar L, Kumarasamy Y. Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the in vitro antibacterial screening of phytochemicals. Methods. 2007 Aug;42(4):321-24. [**PMID**: 17560319]

[18] Huyen HM, Huy VT, Hoa TTT. Antimicrobial activity of herbal extracts against shrimp pathogenic bacteria. Can Tho University of Journal Science. 2018 Jul 30;54(2):143-50.

[19] Mostafa AA, Al-Askar AA, Almaary KS, Dawoud TM, Sholkamy EN, Bakri MM. Antimicrobial activity of some plant extracts against bacterial strains causing food poisoning diseases. Saudi J Biol Sci. 2018 Feb;25(2):361-66. [**PMID**: 29472791]

[20] Canillac N, Mourey A. Antibacterial activity of the essential oil of Picea excelsa on Listeria, *Staphylococcus aureus* and coliform bacteria. Food Microbiology. 2001 Jun 1;18(3):261-68.

[21] Arun T, Senthilkumar B, Purushothaman K, Aarthy A. GC-MS determination of bioactive components of *Phyllanthus amarus* (L.) and its antibacterial activity. J Pharm Res. 2012;5:4767-71.

Keywords: EMS; AHPND; Herbs; Extract; *Phyllanthus amarus*; MIC/MBC