CONTENT

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Antimicrobial resistance of *Escherichia coli* causing edema disease in post-weaning pigs in Vinh Long province

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ABSTRACT

The study was conducted to determine the antimicrobial resistance of Escherichia coli which causes edema disease in post-weaning pigs in Vinh Long province. The results showed that 150 out of 769 examined piglets were positive with E. coli causing edema disease (19.51%). Among the infected piglets, the mortality rate of the disease was 57.33% (86/150). The proportion of E. coli infection in weaned pigs was higher on 1-2 weeks post weaning (66.95%) compared to over twoweeks of post weaning (33.05%). The proportion of E. coli isolated from piglets at households was significantly higher than that at the farms (61.02% vs 38.98% respectively). The most common symptoms of E. coli infection in piglets were swollen eyelids (100%), followed by swollen head (83.90%), and convulsions (82.20%). Moreover, the lesions including fluid accumulation in abdominal cavity (97.67%), hemorrhage in mesentery (96.51%) and small intestines (96.51%) were frequently detected in infected piglets. Positive rate of gene encoding virulent factor Stx2e in post-weaning pigs was quite high (42.37%). E. coli strains were highly resistant to Ampicillin (92%) and Bactrim (77%), followed by Streptomycin (68%) and Gentamycin (50%). The 117/118 strains were multi-resistant to 2-7 antibiotics with 66 different diversified and complicated types. E. coli was still sensitive to ceftazidime, cefuroxime, amikacin, and amoxicillin-clavulanic acid.

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1 INTRODUCTION

Pig production is an important food industry providing high nutrient for humans. As pig production is developed more and more that increased breed demand, diversified and diseases occurred more complicated. The remaining large percentage of small pig farms leads to usual occurrence of diseases, e.g. edema disease caused by *Escherichia coli*.

E. coli strains producing Stx2e toxin which was important virulent caused edema disease in post-weaning pigs. The most common strains of *E. coli* which caused edema disease with high morbidity and mortality rate were F4 (K88) and F18 (Zimmerman *et al.*, 2012). Nguyen Thi Thanh Hoa *et al.* (2010) showed that pigs in small scale farms in Phu Tho had edema disease in high morbidity (28.06%) and mortality rate (56.47%). Farmers used several antibiotics which were added to feed and drinking water for disease prevention. However, abuse of antibiotics in pig production might lead to antibiotic resistance. According to Uemura *et al.* (2004), increase of antibiotic resistance of *E. coli* causing edema disease was deserved well concern

and should be studied more. In Vietnam, the antibiotic resistance in animal production became a matter of social concern for human and animal health. Nguyen Thi Kim Lan (2003) reported that *E. coli* causing edema diseases in weaned pigs in Thai Nguyen province was resistant to ampicillin and cefuroxime. Vinh Long province has a big herd of swine. Edema disease caused by *E. coli* is commonly occurred in post-weaning pigs, but treatment was often ineffective and economic loss for farmers. Therefore, study on antimicrobial resistance of *E. coli* causing edema disease in post-weaning pigs was essential for animal productions. The study was conducted to determine the edema morbidity rate, virulent factor of Stx2e, common strains F4, F18 and antibiotic resistance of *E. coli* causing edema in post-weaning pigs in Vinh Long province.

2 MATERIALS AND METHODS

2.1 Materials

One hundred and eighteen feces samples and 86 mesenteric lymph nodes of post-weaning piglets (from 769 examined piglets) were collected from 12 farms and 21 households at four four districts in Vinh Long province from August 2016 to February 2018.

The following antibiotic discs were used: amoxicillin/clavulanic acid (Ac) 20/10 µg, colistin (Co) 10 µg, gentamicin (Ge) 10 µg, amikacin (Ak) 30 µg, streptomycin (Sm) 10 µg, tetracycline (Te) 30 µg, doxycycline (Dx) 30 µg, ampicillin (Am) 10 µg, trimethoprim-sulfamethoxazole (bactrim) (Bt) 1.25/23.75 µg, ceftazidime (Cz) 30 µg, cefuroxime (Cu) 30 µg, and levofloxacin (Lv) 5 µg (Nam Khoa Co. Ltd, Vietnam).

Materials used for Polymerase chain reaction (PCR): Forward and reverse primers of F4, F18 and Stx2e (Integrated DNA Technologies, USA) (Table 1); PCR Kit, Go Taq® Green Master Mix, 2X (Promega, USA); 100 bp (Gel loading buffer - Invitrogen) (Promega, USA).

2.2 Methods

2.2.1 Sampling methods

Sampling method was performed according to the TCVN 10782: 2015 (ISO 13307: 2013) (DAH, 1990). Twenty-five grams of feces samples and two mesenteric lymph nodes were collected from piglets having edema disease. In each farm and household, samples were collected from 2-3 piglets/litter from 2-3 herds then stored at cold condition.

2.2.2 Isolation and identification of antimicrobial resistance of E. coli

E. coli was isolated according to Barrow and Feltham (2003) and TCVN 5155-90 (DAH, 2015). Disc diffusion method was performed to test antimicrobial resistance of *E. coli* (Bauer *et al.,* 1966). Zones of growth inhibition were evaluated in accordance with Clinical Laboratory Standards Institute standards (CLSI, 2016) when adequate reference values were available.

2.2.3 Methods of F4, F18 E. coli strains identification and virulent gene Stx2e examination

DNA extraction

DNA of *E. coli* was extracted by heat shock as described by Cerna *et al.* (2003) and Botteldoorn *et al.* (2003). Process of PCR was done following the protocol of Promega Company, USA.

Table 1: List of primers for detection of *E. coli* F4, F18 strains and virulent encoding gene Stx2e of *E. coli* causing edema disease in post-weaning pigs using in PCR

Virulence	Primer sequence (5′–3′)	Amplified product size (bp)	Reference
F4 (*faeG)*	GAA TCT GTC CGA GAA TAT CA GTT GGT ACA GGT CTT AAT GG	499	Boerlin *et al.,* 2005
F18 (*fedA)*	TGG TAA CGT ATC AGC AAC TA ACT TAC AGT GCT ATT CGA CG	313	Boerlin *et al.,* 2005
Stx2e	CCT TAA CTA AAA GGA ATA TA CTG GTG GTG TAT GAT TAA TA	230	Fratamico *et al.*, 2004

2.3 Statistical analysis

Chi-square test with statistical significance set at the 95% confidence level ($p<0.05$) was used for statistical comparisons of prevalence, Chi-square was used for comparison of proportions.

3 RESULTS AND DISCUSSIONS

3.1 Results of the survey on edema disease in post-weaning pigs in Vinh Long province

The results of the survey on edema disease in post-weaning pigs was shown in Table 2.

Out of 769 examined post-weaning pigs of 33 herds at 4 districts in Vinh Long province, 150 edema disease piglets were examined. The morbidity rate of edema disease piglets was 19.51%. Bertschinger *et al.* (1992) reported that the morbidity rate ranged from 30-40% and may reach to 80%. The morbidity rate of edema disease in post-weaning pigs in Vinh Long was lower than other studies. In the survey in Vinh Long province, the reason might be due to the instability of animal production, great reduced prices causing herds in the region decreased, farmer restricted to give feed to piglets. While edema disease often occurred in big, greedy piglets leading over protein intake which serve a good environment for *E. coli* to develop in intestine then cause edema disease (Carlton *et al.*, 2010). The morbidity rate of edema disease in piglets in Vung Liem district (22.97%), was similar to Mang Thit (21.08%), Long Ho (18.41%) and Tra On district (14.29%). (P = 0.337). This might be due to that the four districts are not far from each other, and pigs were traded across the province. The mortality rate was high (57.33%); it is similar to Nguyen Thi Kim Lan's (2003) study which reported that the edema mortality rate of piglets in Binh Dinh province was 61.44%. Zimmerman *et al.* (2012) suggested that the mortality rate ranged from 50% to 90% of edema disease in piglets. The mortality rate at 4 districts was very significant difference (P = 0.000). Those differences might be due to different knowledge and diseases prevention procedures that affected to mortality rate in those areas.

Table 2: Results of the survey on edema disease in post- weaning pigs in Vinh Long province

Location (district)	Herd	No. of examined samples	Morbidity		Mortality	
			Number	(%)	Number	(%)
Tra On	12	161	23	14.29	12	52.17
Vung Liem	7	222	51	22.97	6	11.76
Long Ho	7	201	37	18.41	34	91.89
Mang Thit	7	185	39	21.08	34	87.18
				P=0.337		*P=0.000*
Total	33	769	150	19.51	86	57.33

3.2 Result of isolation of *E. coli* causing edema in post-weaning pigs in Vinh Long province

The result of isolation of *E. coli* causing edema in post-weaning pigs was shown in Table 3, 4 and 5.

There were 118/118 feces samples and 86/86 lymph node samples from 118 edema disease piglets were positive with *E. coli* in ratio of 100%. These results contributed to specifying edema disease piglets caused by *E. coli* with typical symptoms. According to Carlton *et al.* (2010), *E. coli* invaded to the epithelial cells of the intestinal wall by various adhesive factors, entered the mesenteric lymph nodes through the lymphatic system and secreted Stx2e toxin, then the toxin entered the blood stream and located in different organs. Therefore, the isolation rate of *E. coli* in colonic mesentery lymph nodes was 100%.

Table 3: Result of isolation of *E. coli* causing edema in post-weaning pigs in Vinh Long province

Location (district)	Feces samples			Mesenteric lymph nodes		
	No. of isolates	No. of positive	Rate (%)	No. of isolates	No. of positive	Rate (%)
Tra On	25	25	100	12	12	100
Vung Liem	25	25	100	6	6	100
Long Ho	34	34	100	34	34	100
Mang Thit	34	34	100	34	34	100
Total	118	118	100	86	86	100

The positive rate of *E. coli* isolation in post-weaning pigs about two weeks after weaning (66.95%) was higher than that over two weeks (33.05%), and there was statistically significant difference (P<0.01). In the first stage of 1-2 weeks, weaned pigs might confront sudden changes such as temperature, humidity, barns, feed, separating or putting in herd, as well as immature digestion and immune systems. These factors created opportunities for pathogenic agents to invade and cause diseases in piglets. Bertschinger *et al.* (1992) reported that the edema disease often occurred in piglets about two weeks after weaning and 4-12 weeks of age. Therefore, in the post-weaning period, farmers should early detect symptoms of edema disease. Moreover, they should ensure the proper nutrition, appropriate temperature, and regularly keep sanitary and disinfected barns.

Table 4: Result of isolation of *E. coli* causing edema disease in post-weaning pigs in Vinh Long province by age

After weaning (week)	Positive results	Rate (%)
1 – 2	79	66.95
> 2	39	33.05
		P=0.000
Total	118	100

Table 5: Result of isolation of *E. coli* causing edema disease in post-weaning pigs in Vinh Long province by farm types

Types	Examined herds	No. of positive samples	Rate (%)
Household	21	72	61.02
Farm	12	46	38.98
			P= 0.001
Total	33	118	100

The morbidity rate of edema disease in post-weaning pigs in household with 61.02% were higher than that in farms (38.98%). There was statistically significant difference between two farm types (P<0.01). In this survey, farmers in industrial farms had raising and caring techniques, and knowledge on disease prevention. Conversely, farmers in household had limited on technical rising and caring such as periodic vaccination, frequent barn. The risky factors lead to edema disease in piglets caused by *E. coli*. Thus, the farm types were significantly impacted to morbidity rate of edema disease in post-weaning pigs. Furthermore, susceptibility of piglets to edema disease depended on several factors, especially on genetic resistance, feeding, and immunity (Bertschinger and Gyles, 1994).

3.3 Results of symptoms and lesions observation of edema disease caused by *E. coli* in post-weaning pigs in Vinh Long province

The symptoms and lesions observation of edema disease caused by E. coli in post-weaning pigs were shown in Table 6 and 7.

Table 6: Typical symptoms frequently occurred on edema disease causing by *E. coli* in post-weaning pigs in Vinh Long province

Symptoms	Frequency of occurrence	Rate (%)
Swelling of the eyelids	118	100.00
Moved convulsions	99	83.90
Swelling of the head	97	82.20
Hoarse sound	85	72.03
Diarrhea	62	52.54
		P=0.000

There were typical symptoms in piglets infected with edema disease that were observed in Vinh Long province. Swelling of the eyelids was the most common typical symptoms frequently occurred in edema disease of post-weaning pigs (100%), followed by convulsions (83.90%), swelling of the head (82.20%), hoarse sound (72.03%), and the least common symptom was diarrhea (52.54%). There was statistically significant difference among the symptoms (P<0.01). Similarly, Zimmerman *et al.* (2012) described edema occurrence in the head, eyelids, laryngeal swelling which causes hoarse sound, nervous signs as crush headlong into the wall, walking around and convulsion. According to Carlton *et al.* (2010), fever or/and diarrhea might not show in edema disease.

Table 7: Typical lesions frequently occurred on edema disease caused by *E. coli* in post-weaning pigs in Vinh Long province

Lesions	Frequency of occurrence	Rate (%)
Fluid accumulate in abdominal cavity	84	97.67
Small intestinal hemorrhage	83	96.51
Mesenteric hemorrhage	83	96.51
Mesenteric lymph nodes swelling	82	95.35
Fluid accumulation in chest cavity	80	93.02
Pericardia swelling	70	81.40
Stomach swelling and hemorrhage	62	72.09
		P=0.000

Of the 86 dead piglets caused by edema disease, the proportion of fluid accumulation in the abdominal cavity was highest with 97.67%, followed by small intestinal hemorrhage, mesenteric hemorrhage, mesenteric lymph nodes swelling, fluid accumulate in chest cavity, pericardium swelling, stomach swelling, and hemorrhage with 96.51%, 96.51%, 95.35%, 93.02%, 81.40% and 72.09%, respectively (P <0,01). These lesions were frequently observed in piglets infected with edema disease in Vinh Long province. Zimmerman *et al.* (2012) showed that the most obvious lesions were gastric hemorrhage, intestinal hemorrhage, fluid accumulated in chest cavity and abdomen, and brain edema.

3.4 Results of identification of F4, F18 strains and virulent encoding gene Stx2e of *E. coli* causing edema disease in post-weaning pigs in Vinh Long province

The F4, F18 strains and virulent gene Stx2e of *E. coli* causing edema disease in post-weaning pigs were identified in Table 8.

Strain F18 with the highest rate (59.32%) were identified in 70 samples, and the lowest were F4 with 20.34% (24/118 samples). The results showed that F18 was the most common strains causing edema disease in post-weaning pigs in Vinh Long province. Zimmerman *et al.* (2012) suggested that F18 was the dominant strain in edema disease piglets. Gene encoding virulent factor Stx2e of *E. coli* causing edema disease in post-weaning pigs was 42.37%. The distribution of Stx2e gene was different among four districts with P = 0.000. It might be due to the numbers of dead piglets in Mang Thit and Long Ho was higher than those in Vung Liem and Tra On (Table 2). *E. coli* invaded to the epithelium of the intestinal wall by various adhesive factors then moved to the mesenteric lymph nodes through the lymphatic system and secreted Stx2e toxin (Carlton *et al.*, 2010). Thus, the rate of gene encoding virulent factor Stx2e from dead piglets was higher. In addition, Botteldoorn *et al.* (2003) showed that the Stx2e gene of *E. coli* strains isolated from edema disease piglets was 31%. This study showed the differences among geographic areas, farm types and samples.

Table 8: Results of identification of F4, F18 strains and Stx2e gene of *E. coli* causing edema in post-weaning pigs in Vinh Long province (n=118)

Places	Genes					
	F4		F18		Stx2e	
	No. of positive samples	Rate (%)	No. of positive samples	Rate (%)	No. of positive samples	Rate (%)
Tra On	8	33.33	16	22.86	4	3.39
Vung Liem	4	16.67	15	21.43	7	5.93
Long Ho	7	29.17	25	35.71	14	11.86
Mang Thit	5	20.83	14	20.00	25	21.19
		P=0.528		*P=0.118*		***P= 0.000***
Total	24	20.34	70	59.32	50	42.37

3.5 Results of antibiotics resistance of *E. coli* strains causing edema disease in post-weaning pigs in Vinh Long province

The result of antibiotics resistance of *E. coli* strains causing edema disease in post-weaning pigs was described in Table 9 and 10.

The prevalence of multidrug-resistant bacteria in poultry and pig production was a public health concern. These bacteria could be transmitted to humans via the food chain or direct contact (Docic and Bilkei, 2003). The result of this study showed that *E. coli* strains isolated from piglets infected with edema disease in Vinh Long province were highly resistant to ampicillin (92.37%), bactrim (77.12%), streptomycin (67.80%), and fairly resistant to gentamicin (50%). In this study, both of F4 and F18 strains showed high resistance to ampicillin (91.67%, 91.43%), moderate resistance to bactrim (70.83%, 75.71%), and streptomycin (66.67%, 64.29%), respectively. *E. coli* causing edema disease in Vinh Long was highly resistant to ampicillin and streptomycin because the two antibiotics were frequently used to treat animal diseases by farmers. Choi *et al*. (2002) reported that *E. coli* isolated from diarrhea and edema disease in post-weaning pigs in Korea were highly resistant to ampicillin (91.19%), tiamulin (91.19%), tylosin (90.70%), and trimethoprim/sulfamethoxazole (87.21%). The antibiotic resistance of *E. coli* in this study were higher than that in other studies by Okello *et al.* (2015) with ampicillin (41.00%), by Bessone *et al.* (2017) with ampicillin (61.90%), bactrim (41.37%), and gentamycin (9.52%). These results showed that antibiotic resistance was increased and diverse in various areas

Table 9: Result of antibiotics resistance of E. coli F4, F18 strains causing edema disease in post weaning pigs in Vinh Long province

Antibiotics	Total (n=118)		F4 (n=24)		F18 (n=70)	
	Resistance (%)	Susceptibility (%)	Resistance (%)	Susceptibility (%)	Resistance (%)	Susceptibility (%)
Ampicillin	92.37	7.63	91.67	8.33	91.43	8.57
Bactrim	77.12	22.88	70.83	29.17	75.71	24.29
Streptomycin	67.80	32.20	66.67	33.33	64.29	35.71
Gentamycin	50.00	50.00	54.17	45.83	47.14	52.86
Levofloxacin	47.46	52.54	45.83	54.17	38.57	61.43
Tetracycline	34.75	65.25	41.67	58.33	34.29	65.71
Colistin	31.36	68.64	33.33	66.67	30.00	70.00
Doxycycline	24.58	75.42	20.83	79.17	22.86	77.14
Ceftazidime	3.39	96.61	4.17	95.83	4.29	95.71
Cefuroxime	2.54	97.46	4.17	95.83	4.29	95.71
Amikacin	0.85	99.15	0	100.00	0	100.00
Amoxicillin-Clavulanic acid	0	100.00	0	100.00	0	100.00

Table 10: Multidrug resistance of *E. coli* strains causing edema disease in post-weaning pigs in Vinh Long province

No. of antibiotics	Phenotypes resistance	No. of multi-resistant types	No. of phenotypes / 117 strains tested	Rate (%)
2	Am+Bt	7	6	5.13
	Am+Ge		3	2.56
	Am+Lv		2	1.71
	Am+Sm		1	0.85
	Am+Sm		1	0.85
	Lv+Bt		1	0.85
	Sm+Lv		1	0.85
Subtotal			*15*	*12.82*
3	Am+Co+Bt	11	1	0.85
	Am+Co+Dx		1	0.85
	Am+Dx+Bt		1	0.85
	Am+Lv+Bt		4	3.42
	Am+Sm+Bt		7	5.98
	Am+Sm+Ge		3	2.56
	Am+Te+Bt		1	0.85
	Ge+Lv+Bt		1	0.85
	Lv+Te+Bt		1	0.85
	Sm+Ge+Bt		1	0.85
	Sm+Ge+Lv		1	0.85
Subtotal			*22*	*18.80*
4	Am+Co+Dx+Te	13	1	0.85
	Am+Ge+Dx+Lv		1	0.85
	Am+Ge+Lv+Bt		1	0.85
	Am+Sm+Co+Bt		2	1.71
	Am+Sm+Cz+Bt		1	0.85
	Am+Sm+Dx+Bt		1	0.85
	Am+Sm+Ge+Bt		7	5.98
	Am+Sm+Ge+Lv		3	2.56
	Am+Sm+Ge+Te		1	0.85
	Am+Sm+Lv+Bt		2	1.71
	Am+Sm+Lv+Te		1	0.85
	Am+Sm+Te+Bt		2	1.71

No. of antibiotics	Phenotypes resistance	No. of multi-resistant types	No. of phenotypes / 117 strains tested	Rate (%)
	Ge+Co+Dx+Te		1	0.85
Subtotal			*24*	*20.51*
5	Am+Co+Dx+Te+Bt	18	1	0.85
	Am+Co+Lv+Te+Bt		2	1.71
	Am+Ge+Co+Dx+Bt		1	0.85
	Am+Ge+Co+Dx+Te		1	0.85
	Am+Ge+Co+Lv+Bt		1	0.85
	Am+Ge+Cz+Lv+Bt		1	0.85
	Am+Ge+Dx+Te+Bt		1	0.85
	Am+Sm+Co+Dx+Bt		1	0.85
	Am+Sm+Co+Lv+Bt		3	2.56
	Am+Sm+Co+Te+Bt		3	2.56
	Am+Sm+Dx+Te+Bt		1	0.85
	Am+Sm+Ge+Ak+Lv		1	0.85
	Am+Sm+Ge+Co+Bt		4	3.42
	Am+Sm+Ge+Dx+Bt		1	0.85
	Am+Sm+Ge+Dx+Lv		2	1.71
	Am+Sm+Ge+Lv+Bt		1	0.85
	Am+Sm+Ge+Te+Bt		1	0.85
	Am+Sm+Lv+Te+Bt		2	1.71
Subtotal			*28*	*23.93*
6	Am+Co+Dx+Lv+Te+Bt	11	1	0.85
	Am+Sm+Co+Dx+Te+Bt		1	0.85
	Am+Sm+Co+Lv+Te+Bt		3	2.56
	Am+Sm+Dx+Lv+Te+Bt		2	1.71
	Am+Sm+Ge+Co+Dx+Lv		1	0.85
	Am+Sm+Ge+Co+Te+Bt		1	0.85
	Am+Sm+Ge+Cz+Cu+Bt		1	0.85
	Am+Sm+Ge+Dx+Lv+Bt		1	0.85
	Am+Sm+Ge+Dx+Te+Bt		1	0.85
	Am+Sm+Ge+Lv+Te+Bt		3	2.56
	Sm+Ge+Co+Lv+Te+Bt		1	0.85
Subtotal			*16*	*13.68*
7	Am+Ge+Co+Dx+Lv+Te+Bt	6	2	1.71
	Am+Sm+Ge+Co+Dx+Lv+Bt		1	0.85
	Am+Sm+Ge+Co+Lv+Te+Bt		3	2.56
	Am+Sm+Ge+Cu+Lv+Te+Bt		1	0.85
	Am+Sm+Ge+Cz+Cu+Lv+Bt		1	0.85
	Am+Sm+Ge+Dx+Lv+Te+Bt		4	3.42
Subtotal			*12*	*10.26*
Total		***66***	*117*	*100.00*

E. coli causing edema in post-weaning pigs in Vinh Long province was multidrug resistant to 2-7 antibiotics with 66 phenotypes which were diversified and complicated. In which, 18 multi-resistant phenotypes to five antibiotics had the highest percentage (23.93%), followed by 13 phenotypes (20.51%) to four antibiotics; 11 phenotypes (18.8%) to three antibiotics; 11 phenotypes (13.68%) to six antibiotics. The result of this study showed that *E. coli* causing edema disease in post-weaning pigs in Vinh Long province was multidrug resistant to many antibiotics.

4 CONCLUSIONS

The morbidity proportion of edema disease in post-weaning piglets in Vinh Long province was 19.51%, and the mortality rate was 57.33%. Edema disease in post-weaning pigs was age-dependent. F4 and F18 strains of *E. coli* caused edema disease in post-weaning pigs in Vinh Long; and F18 was the most common strain (59.32%). Stx2e toxin caused high edema mortality rate in post-weaning pigs (42.37%).

E. coli strains of F4 and F18 isolated from edema piglets were highly resistant to ampicillin, bactrim, streptomycin, moderately resistant to gentamycin. *E. coli* strains were multidrug resistant to 2-7 antibiotics with very diversified and complicated phenotypes.

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Study on effectiveness of activated charcoal and wood vinegar on prevention of piglet diarrhea

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ABSTRACT

The study was conducted from January 2016 to July 2016 at some pig farms in Can Tho City to determine the effectiveness of activated charcoal and wood vinegar on protecting piglet from diarrhea. The experiment was set in 19 herds of suckling pig (217 piglets) and 21 herds of post weaning pig (226 piglets) with activated charcoal: wood vinegar in 8 g:2 ml/1kg feed. The result showed that average ratio of diarrhea in suckling pigs (7.37%) was higher than that in post weaning pigs (1.32%) in experimental group; but these rates were deep descended in comparison with control groups (27.94% and 30.3% respectively). Applying activated charcoal and wood vinegar in feed was effective to prevent diarrhea in suckling pigs and post weaning pigs in both rainy and dry seasons as 2.77%, 4.54% and 3.77%, 5.88% in comparison with control groups 36.36%, 16.67% and 54.54%, 25.45%, respectively. However, such an application was not effective to weight gain of piglets at 60 days old (16.827kg) and in control group (15.327 kg), neither to feed conversion ratio 1.474 and 1.592, respectively. The experimental herds were not used antibiotic when piglet having diarrhea recovered themselves after one day, usefully increasing income of farmers (124%).

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1 INTRODUCTION

Livestock production constitutes a very important component of the agricultural economy of developing countries; especially swine production plays an important role in meat production to human. Swine production in the Mekong Delta is currently common in small size with open pen where herds' health is easily affected by risk factors such as temperature, humidity and diseases (Hong *et al.*, 2006). Among common diseases, piglet diarrhea, especially in suckling and post weaning pigs, causes economic losses to farmers. There are many causative agents leading to diarrhea in piglets such as microorganisms, host immunity and caring process (Katsuda *et al.*, 2006). *Escherichia coli* appeared to be the most dangerous agents causing diarrhea in piglets (Callesen *et al.*, 2007).

Nowadays, antibiotic abuse for the treatment of diarrhea in piglets leads to antibiotic resistance of *E. coli* strains (Hunter *et al.*, 2010). Antibiotic abuse is not only generating more antibiotic resistant microorganisms, but also influencing consumers' health. Thus, reduction of antibiotic use becomes overall trend in the world and in Vietnam. Currently,

it is necessary to find the bio-products for antibiotic replacement that do not affect human and animal health. Activated charcoals are known in absorption capability to not only harmful bacteria in digestive tract such as *Salmonella* and *E. coli*, but also bacteria toxic secretion in vitro and in vivo experiments a long time ago (Naka *et al.,* 2000). Besides activated charcoal, wood vinegar or organic acidifier compounds were used for supplying young animals in digestive sterilization, increasing protein digestion, and stimulating useful microbe duplication in gastrointestinal tract (Partanen and Mroz, 1999). Moreover, almost industry farms and small-scale farms in the Mekong Delta have been intensified on farms hygiene, disease prevention, and vaccination. However, diseases continuously occur with high frequency while antibiotic treatment has low effectiveness. Therefore, the study was conducted to evaluate the effectiveness of activated charcoal, wood vinegar on prevention of piglet diarrhea caused by *E. coli* and to determine antibiotic alternatives in prevention and treatment of digestive diseases in piglets.

2 MATERIALS AND METHODS

2.1 Materials

Two hundred and seventeen suckling piglets (1-28 days old), 226 post-weaning pigs (28-60 days old) with neonatal weight range of 1.418 – 1.440 kg were selected, which belong to Landrace x Yorkshire x Duroc of second litter sows in swine farms in Can Tho City, suckling piglet was fed at 7 days of age with diet of activated charcoal mixed with wood vinegar. Antibiotics such as enrofloxacin, amoxicillin, colistin, norfloxacin, were injected intramuscularly for diarrhea treatment in 3-5 days. Vitamin C, B complex and glucose were applied in oral to 5 days into 2 groups. Activated charcoal was bought from Xuyen Viet Environment *Ltd., Co*. at Ho Chi Minh city, and wood vinegar was produced by laboratory of Specified Veterinary Medicine, CTU.

2.2 Experimental design for evaluation of effectiveness of activated charcoal and wood vinegar on prevention of piglet diarrhea

The evaluation of effectiveness of activated charcoal and wood vinegar on prevention of piglet diarrhea was arranged in Table 1.

Table 1: Experimental design

Parameter	Suckling Pigs		Post Weaning Pigs	
	Experimental	Control	Experimental	Control
Number of Experimental farms	4	4	3	3
Total herds	19	6	21	6
Average number of piglets/litter	11	11	11	11
Suckling Pig Feed	Apollo Cargill	Apollo Cargill	ApolloCargill	ApolloCargill
Weaned Pig Feed	Windmill 3120	Windmill 3120	Windmill 3120	Windmill 3120
Ratio AC:WV/1kg Feed	8:2	0	8:2	0
Feeding way	Mixed feed		Mixed feed	

AC: activated charcoal, WV: wood vinegar. Proportion 8:2: 8g activated charcoal: 2ml wood vinegar.

2.3 Evaluation of diarrhea preventing efficiency

Diarrhea preventing efficiency was measured by formulas of diarrhea ratio, average diarrhea ratio and seasonal diarrhea ratio as follows:

$$\text{Diarrhea ratio (week)} = \frac{\text{the number of weekly diarrhea piglet}}{\text{the number of weekly surveyed piglet}} \text{X100}$$

$$\text{Average diarrhea ratio} = \frac{\text{the number of average diarrhea piglet}(period)}{\text{the number of surveyed piglet (period)}} \text{X100}$$

$$\text{Seasonal diarrhea ratio} = \frac{\text{the number of average diarrhea piglet(season)}}{\text{the number of surveyed piglet (season)}} \text{X100}$$

2.4 Evaluation of feed consuming efficiency

Feed consuming efficiency was measured by formulas as follows:

$$\text{Average daily feed intake (ADFI)} = \frac{\text{amount of feed intake (period)/ total days}}{\text{total piglets of litter}}$$

$$\text{Average daily gain (ADG)} = \frac{\text{average weight gain (period)}}{\text{number of day (period}}$$

$$\text{Feed conversion ratio (FCR)} = \frac{\text{total number feed consumed (period)/total piglets of litter}}{\text{average weight gain (period)}}$$

2.5 Weight gain performance evaluation

Weight of suckling pig was measured in the early morning, before feeding at day 1, 7 and 28. Weight of post weaning pig was measured at day 28 and 60.

2.6 Economic efficiency comparison

Economic efficiency of experimental treatment was evaluated by gap between total income and total outcome of each period: Gap = total outcome– total income.

2.7 Statistical analysis

The data were analyzed by Chi-square and general linear model using Minitab 16.0 software.

3 RESULTS AND DISCUSSIONS

3.1 Result of diarrhea rate observation of piglets in experimental treatment

The diarrhea rate observation of piglets in experimental treatment was shown in Table 2.

Table 2: The proportion of piglet diarrhea in experimental treatment

Period	No. of farms	No. of herds	No. of piglets	Average No. of diarrheic piglets	Ratio (%) [Treatment/Control]
Suckling	4	19	217	16	7.37/27.94
Post weaning	3	21	226	3	1.32/30.30
					P=0.002

The ratio of suckling pigs with diarrhea was 7.37% which was higher than that of post weaning pig (1.32%) (P<0.01). The different rates might be due to low resistance of suckling pigs and mostly dependence on passive antibody receiving from colostrum. These passive antibodies have been decreased in very low level at fourth week of age (Zivkovic and Kovcin, 1989). Antibody production in suckling pig had just activated in this period compared to post weaning pig with nearly immune completion. In the other hand, suckling pig has an immature digestive tract, consuming a large amount of feed, low HCl level in stomach, low capable protein digestion creating favorable conditions for *E. coli* growth which caused piglet diarrhea. In contrast, gastrointestinal tract (GI) of post weaning pig is completely developed with sterilized capability by increasing amount of free HCl, therefore, the ratio of diarrhea in weaned pig is lower than that in suckling pig when charcoal and wood vinegar were added (Zivkovic and Kovcin, 1989).

3.2 The proportion of diarrheic piglets in the groups by period of time

The proportion of diarrheic suckling pigs by period of time was shown in Table 3.

Table 3: The diarrhea rate of suckling pigs in two groups by period

Week	Experimental treatment			Control group			P
	No. of surveyed piglets	No. of diarrheic piglets	Ratio (%)	No. of surveyed piglets	No. of diarrheic piglets	Ratio (%)	
1	217	13	5.99	68	4	5.88	0.79
2	217	44	20.20	68	24	35.29	0.01
3	217	6	2.76	68	23	33.82	0.00
4	217	2	0.92	68	25	36.76	0.00

The diarrhea rate of suckling pigs in the experimental and control groups at the first week were 5.99% and 5.88%, respectively, and there was no significant difference with P=0.79. The result was probably explained that in this period, all piglets of two treatments did not consume any feed, so there is no significant difference. When piglets started consuming feed in the second week, the diarrhea rate in the second, third, fourth week of the experimental group was 20.2%, 2.76%, 0.92%, respectively; that were completely lower than those of the control group with 35.29% (P<0.005), 33.82% and 36.76%, respectively (P<0.001). These results showed the similarities with the report by Naka *et al.* (2000) that providing activated charcoal and wood vinegar in feed reduced diarrhea rate by absorption of activated charcoal to *E. coli* in digestive system. The older piglets at three and four weeks of age consumed more feed, thus the diarrhea prevention capability of activated charcoal and wood vinegar was obviously demonstrated in the experimental group compared to the control group.

The proportion of diarrheic post-weaning pigs by period of time was shown in Table 4.

Table 4: The diarrhea rate of post-weaning pigs in two groups by period of time

Week	Experimental Treatment			Control group			P
	No. of surveyed piglets	No. of diarrheic piglets	Ratio (%)	No. of surveyed piglets	No. of diarrheic piglets	Ratio (%)	
5	226	5	2.12	66	21	31.81	0.000
6	226	4	1.76	66	32	48.48	0.000
7	226	1	0.44	66	15	22.72	0.000
8	226	3	1.32	66	11	16.67	0.000

The ratios of post weaning pig diarrhea at fifth, sixth, seventh and eighth week were 2.12%; 1.76%; 0.44% and 1.32%, respectively that were highly significant difference (P<0.01) when compared to the control group in 31.81%, 48.48%, 22.72% and 16.67%, respectively. These results might be due to post weaning pigs depended on feed and amount of feed increased by age after separated far from sows. In the control group, the amount of feed intake rose while indigestible feed amount increased. It is a good condition for multiplication of *E. coli, Salmonella*, stress caused by weaning and change of feed, leading to easily effected low digestive feed ability (Thomson, 2006). In the experimental group, there was the presence of activated charcoal and wood vinegar, which killed bacteria efficiently to assist pigs in digestive feed, activated stomach pepsin and stimulated beneficial bacteria to develop and compete with *E. coli* for attachment on gut receptor; when *E. coli* cannot attach to gut receptor, it lost capability to cause diarrhea (Watarai and Tana, 2005).

Table 5: The diarrhea rate in suckling pigs of two groups by seasons

Season	Experimental treatment			Control group			P
	No. of surveyed piglets	No. of diarrheic piglets	Ratio (%)	No. of surveyed piglets	No. of diarrheic piglets	Ratio (%)	
Dry	159	6	3.77	42	7	16.67	0.003
Rainy	36	1	2.77	11	4	36.36	0.007

The rates of suckling pig diarrhea in the experimental and control groups in dry and rainy seasons were 3.77% and 16.67%; 2.77% and 36.36%, respectively. This result indicated that the ratios of diarrhea in suckling pigs in the control group were higher than those in the experimental group in both dry and rainy seasons (P<0.01). In the control group, the diarrhea rate was increased when climate changes from dry to rainy season. Supplementing activated charcoal and wood vinegar in suckling pig diet of the experimental group reduced the ratio of piglet diarrhea even under temperature and humidity factors. Supplying activated charcoal and wood vinegar played an important role in piglet immunity and digestibility in different weather conditions. As diets of suckling pigs were supplemented with activated charcoal and wood vinegar which continued to increasing effective impact on decimating harmful bacteria and stimulating useful bacteria's growth in piglet GI tract (Naka *et al*., 2000).

Table 6: The diarrhea rate in post weaning pigs in two groups by seasons

Season	Experimental treatment			Control group			P
	No. of surveyed piglets	No. of diarrheic piglets	Ratio (%)	No. of surveyed piglets	No. of diarrheic piglets	Ratio (%)	
Dry	204	12	5.88	55	14	25.45	0.000
Rainy	22	1	4.54	11	6	54.54	0.002

In the same suckling pigs, diarrhea rates in post weaning pigs of the experimental and control groups in dry and rainy seasons were 5.88% and 25.45%; 4.54% and 54.54%, respectively. There was significant difference between diarrhea rates of the experimental and control groups in both dry and rainy seasons (P<0.01). The efficiency of supplementing activated charcoal and wood vinegar for piglet diet proved piglet ability in reducing impact of humidity and temperature to diarrhea rate of suckling and weaned piglets.

3.3 Results of treatment of piglet diarrhea

The results of treatment of piglet diarrhea was shown in Table 7 and Table 8.

In the control group, 76 diarrhea piglets (100%) treated by antibiotic recovered in one to five days (17 out of 76 piglets recovered after five days), while suckling pigs at the experimental treatment,52 out of 65 piglets with slight diarrhea (80%) were not treated by antibiotic and self-recovered after one day, 13 out of 65 piglets (20%) were treated by

antibiotic. Because at the first week of age, piglets did not consume feed supplemented with activated charcoal and wood vinegar, there was no effect in diarrhea prevention.

In the experimental group, there were 13 diarrheic piglets recovering themselves after one day. It reveals that activated charcoal and wood vinegar showed effectiveness in diarrhea prevention. In the control group, 10 out of 79 piglets with diarrhea recovering after one day, 69out of 79 piglets recovering after 2-5 days, were treated by antibiotics. It means that activated charcoal and wood vinegar showed effectiveness in preventing and reducing diarrhea rate in post weaning pigs.

Table 7: Result of treatment of suckling pig diarrhea

Parameter	Experimental treatment		Control group	
	No. of piglets	Ratio (%)	No. of piglets	Ratio (%)
Diarrheic piglets	65	7.37	76	27.94
Piglets were not treated with antibiotics	52	80.00	0	0
Piglets were treated with antibiotic	13	20.00	76	100
Recovered day (day)				
1	52	80.00	10	13.15
2	0	0	29	38.15
3	13	20.00	20	26.31

Table 8: Result of treatment of post weaning pig diarrhea

Parameter	Experimental treatment		Control group	
	No. of piglet	Ratio (%)	No. of piglet	Ratio (%)
Diarrheic piglet	13	1.32	79	30.30
Piglets were not treated with antibiotic	13	100		
Piglets were treated with antibiotic	0	0	79	100
Recovered day (day)				
1	13	100	10	12.65
2	0		31	39.24
3	0		35	44.30
5	0		3	3.79

3.4 Results of weight gain, feed consumed efficiency and conversion ratio of piglets

The weight gain, feed consumed efficiency and conversion ratio of piglets were shown in Table 9, 10 and 11.

Results of the study showed that average weights of neonatal piglet in the experimental and control groups were 1.418kg and 1.440kg, respectively. There was similarity in 1-week-old piglet weight of experimental (3.992 kg) and control (4.093 kg) (P=0,204). These consequences could be explained that piglets of each treatment did not consume any feed, required nutrition for their growth almost from sow's milk. From fourth week, average weight of piglets in the experimental group (7.123 kg) was higher than that in the control group (6.681 kg), (P=0,005). The same result was witnessed in eighth week with average piglet weight of the experimental (16.827kg) higher than that of the control group (15.637kg) (P=0.000). In these stages, the ratio of piglet diarrhea in the experimental group which consumed feed mixed with activated charcoal and wood vinegar, was decrease; therefore, nutrient absorption was improved as well as health improvement and weight gain. According to Mekbungwan *et al.* (2004), activated charcoal and wood vinegar were non-toxic ingredients and unaffected in weight gain of piglets, besides they could contribute to the absorption of anti-nutritional factors and toxic in the feed ensuring normal growth of piglets.

Table 9: The results of weight gain of piglets being neonatal to 60 days old

Week	Average weight (kg)		SE	P
	Experimental	Control		
Neonatal	1.418	1.440	0.013	0.255
1	3.992	4.093	0.053	0.204
4	7.123	6.681	0.106	0.005
8	16.827	15.637	0.226	0.000

Table 10: The weight gain parameters in suckling pigs

Parameter	Group		SE	P
	Experimental	Control		
ADG (g/piglet/day)	181.58	175.24	9.877	0.666
ADFI (g/piglet/day)	44.720	40.50	4.187	0.500
FCR	0.462	0.505	0.349	0.414

ADG: average daily gain, ADFI: average daily feed intake

The result showed that ADG and ADFI of piglets in the experimental group were 181.58g and 44.72g, which was similar to these data in the control group with 175.24g and 40.50g (P>0.05). FCR of experimental piglets was 0.462, and there was no statistically significant difference between the experimental and control groups. The data showed that activated charcoal and wood vinegar added to piglet diet did not affected feed consume, DGW and FCR of suckling pig. It means that activated charcoal and wood vinegar used in this experiment were not toxic and did not affect weight gain of suckling pigs.

Table 11: The weight gain in post weaning pigs

Parameter	Group		SE	P
	Experimental	Control		
ADG (g/piglet/day)	301.61	282.76	16.55	0.448
ADFI (g/piglet/day)	448.15	445.63	25.34	0.947
FCR	1.47	1.59	0.04	0.070

In the same suckling pigs, ADG and ADFI of post weaning pigs in the experimental group were 301.61g and 448.15g; which were similar to those data of the control group with 282.76 g and 445.63 g, respectively (P>0,05. FCR of both groups were 1.47 and 1.59, respectively and not statistically significant (P>0.05). These results showed that activated charcoal and wood vinegar mixed into diet were not toxic and did not affect weight gain of post weaning pigs.

3.5 Economic efficiency of two groups

The economic efficiency of two groups were measured Table 12.

Table 12: Comparison of economic efficiency between two groups

Parameter	Experimental treatment	Control group	Price per kg	Total (cost) (Unit: thousand VND	
				Experimental	Control
TF(0-28) (kg)	1.25	1.13	24.72	30.90	27.93
TF(28-60) (kg)	14.34	14.26	14.88	213.30	212.18
TAC0-60 (kg)	1.7	0	14	1.7	0
Diarrhea treated cost				2	46
Total outcome				245.90	286.11
TGW (KG)	16.82	15.63	42	706.44	656.46
Total income				706.44	656.46
Gap between total income and outcome				460.54	370.34
Profit comparison (%)				124%	100%

(TF0-28: Amount of feed period 0-28 day; TF28-60: Amount of feed period 28-60 day; TAC0-60: Amount of activated charcoal provided in feed; TGW: Weight gain period0-60 day)

Economic efficiency was obviously witnessed; profit per pig of experimental treatment was 460.54 thousand VND that was 1.24 times higher than that control group (370.34 thousand VND). In experimental treatment, it was spent about 1.7 thousand VND for activated charcoal and wood vinegar, no cost for diarrhea treatment because of slight diarrhea and self-recovery. Average weights at the end of the experimental group were also higher than those of the control group. However, FCR of the experimental treatment was low so that it brought more profit than the control for farmers. This result indicated that activated charcoal and wood vinegar supplement in diets of piglet contributed to reducing diarrhea rate in both suckling and post weaning pigs. Activated charcoal and wood vinegar added to piglet diet were not toxic and did not affect piglets'

weight gain (Mekbungwan *et al.*, 2004); it was effective to reduce antibiotic use in treatment, reduce production cost, and increase productivity and profit (124%).

4 CONCLUSIONS

Combination of activated charcoal and wood vinegar mixed with feed effectively reduced diarrhea in suckling and post weaning pigs, both in dry and rainy seasons. It did not affect piglet weight gain, FCR, daily feed intake. It helps to restrict the use of antibiotics, increase the quality of products, reduce input costs, and increase profits for farmers (124 %).

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Study on canine respiratory disease and evaluating the effect of treatment at the Animal Clinic, Can Tho University

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ABSTRACT

The research was carried out to investigate the clinical diagnosis in dogs with respiratory tract disease and evaluate the treatment effect in the Animal Clinic of Can Tho University. The determination of respiratory tract disease in dogs was performed by cross-sectional study in 736 dogs reared in Can Tho city. The results showed that 143 out of 736 (19.43%) dogs had signs of respiratory tract disease by the clinical diagnosis. In addition, the respiratory tract disease in dogs was dependent on and gradually increased according to age of dogs (P<0.05); however, it was regardless of sex (P>0.05). The results indicated that dogs had the clinical diagnosis in the upper airways (79.72%) and lower airways (20.28%). Cough combined nasal discharge at the highest rate (18.18%), followed by cough combined nasal discharge and eye rheum (13.99%), increase of respiratory rhythm (13.29%), cough combined nasal discharge and increase of respiratory rhythm (10.49%), nasal discharge combined eye rheum (10.49%), cough combined nasal discharge and rales (9.79%), cough combined rales and depression (6.99%), dry cough (6.29%), cough combined increase of respiratory rhythm and depression (5.59%), nosebleed (2.80%) and nosebleed combined increase of respiratory rhythm (2.10%). The effectiveness of the treatment using Marbofloxacin or Cefuroxime was similar, and the adjustment for base clinical signs of the respiratory tract disease was high or rich in both treatments.

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1 INTRODUCTION

Dog is one of the most dominant pets in Vietnam, as dog is friendly, faithful and useful. According to the Vietnamese, dog is an important pet, local dog is a house guard and has a significant contribution to human life. In recent years, species and number of dogs have increased considerably in Vietnam because of increasing human requirements. Foreign dogs are popularly raised in Vietnam due to a good adaptation to the local climate and feeds. However, dogs nursing is remained a disease challenge and care labeling. Canine respiratory tract disease was considered a common disease syndrome of limited clinical significance. Clinical signs such as cough, nasal discharge and dyspnea are now rarely associated with a single pathogen and more often attributed to multiple agents that sequentially or synergistically cause disease (Priestnall *et al.*, 2014). An important step in the pathogenesis of canine respiratory tract disease involves the colonization of primary respiratory pathogens at the upper airway mucosa. In the susceptible host and the proper environment, these primary respiratory

pathogens are capable of bypassing the mechanical barriers, evading the innate immune response and disrupting mucociliary clearance, thereby allowing both primary and secondary bacterial and viral pathogens to colonize and infect the upper and lower respiratory tract. Many factors contribute to the aetiology of canine respiratory tract disease including viruses and bacteria as well as stress due to mixing and housing in an unfamiliar environment (Erles and Brownlie, 2005). The increase of respiratory tract disease of dogs may seriously influence the dog health. Management as well as early diagnosis of respiratory tract disease in dogs which has an important role in preventing severe complications is necessary for veterinary's work. The main objective of this research was to investigate the clinical diagnosis of canine respiratory tract disease and evaluate the treatment effect of the Animal Clinic of Can Tho University.

2 MATERIALS AND METHODS

2.1 Time and location

This study was conducted in the Animal Clinic, College of Agriculture - Can Tho University, Can Tho City, Vietnam. The implementation of this study was from June to October 2018.

2.2 Animals

Experimental animals were all dogs that infected with respiratory tract disease with typical clinical and atypical clinical manifestations regardless age-, breeds- and sex.

2.3 Equipment and drugs

The equipment such as stethoscope, thermometer and syringe were used in this study, and two currently available antibiotics in the Animal Clinic (Marbofloxacin (10%), Cefuroxime (1 g)) were used to control respiratory tract disease in dogs.

2.4 Methods

2.4.1 Research methods

The clinical diagnosis was used to check the level of respiratory tract disease in dogs. The standard of diagnosis is according to Nguyen Duong Bao (2005) and Pham Ngoc Thach (2008).

2.4.2 Recorded parameters

The rate of dogs with respiratory tract disease on age, breed, sex and rearing modality dependence; the typical clinical and atypical clinical manifestations and evaluation of treatment effect were recorded.

2.4.3 Survey design

The standard for choosing the subjects is dogs with respiratory tract disease, permission and collaboration of owners. The survey design of experiment was presented in Table 1.

Table 1: Layout of experiment

Items	No. (dog)	Medicine
Mar	30	Marbofloxacin with a dose of 1.0 ml/50 kgBW (SC), 1 time/day
Cef	30	Cefuroxime with a dose of 1.0 ml/10 kgBW (SC), 1 time/day

SC: Subcutaneous injection; BW: Body weight

2.4.4 Evaluation of treatment effect

Evaluation of treatment effect was presented in Table 2.

Table 2: Evaluation of treatment effect

Level	Clinical symptom
Good control	No symptoms (no clinical manifestations)
Bad control	Not decreased or died

2.5 Statistical analysis

Mean and standard deviation were calculated using Microsoft Excel version 2016. The data were analyzed by Chi-square of Minitab Statistical Software version 16.0 at the significant level of 5%.

3 RESULTS AND DISCUSSION

3.1 The prevalence of respiratory tract disease in dogs

The study sampling frame comprised 736 dogs attending Animal Clinic. Result on ratio of respiratory tract disease in dogs by random survey in the Animal Clinic of Can Tho University was presented in Table 3.

Table 3: The rate of respiratory tract disease in dogs

No. of examined (dog)	No. of respiratory tract disease (dog)		Items			
			Upper airways		Lower airways	
No.	No.	Percentage (%)	No.	Percentage (%)	No.	Percentage (%)
736	143	19.43	114	79.72	29	20.28

Table 3 showed that the rate of respiratory tract disease in dogs by random survey in the Animal Clinic of Can Tho University was 19.43%. This result was higher than study of Ly Thi Lien Khai (2017), who observed that ratio of respiratory disease in dogs in Can Tho city was 10.49%. The respiratory system consists of the large and small airways and the lungs. When a dog breathes air in through its nose or mouth, the air travels down the trachea, which divides into the tubes known as the right and left bronchi then into the smaller airways called bronchioles in the lungs. The bronchioles end in the small sacs called alveoli (King, 2004; Pham Ngoc Thach, 2008). A varying flora of indigenous commensal organisms (including *Pasteurella multocida, Bordetella bronchiseptica, Streptococci, Staphylococci, Pseudomonas* and *Coliform* bacteria) normally resides in the canine nasal passages, nasopharynx, upper trachea and at least intermittently in the lungs, without causing clinical signs (Kuehn, 1990; Russell *et al.*, 1991; Lappin *et al.*, 2017). Opportunistic infections by these bacteria may occur when respiratory defense mechanisms are compromised by infection with a primary pathogen. Secondary bacterial infections complicate the management of viral respiratory infections of dogs. Pathogens may continue to reside in the respiratory tract of convalescent animals. When stressed, these animals may relapse; they can also act as a source of infection for others. Poor management practices are often associated with poor hygienic and environmental conditions, and the resultant stress increases both the incidence and severity of infections (Kuehn, 1990; Russell *et al.*, 1991; King, 2004).

3.1.1 The prevalence of respiratory tract disease in dogs by ages

Result on respiratory tract disease in dogs by ages was presented in Table 4.

Table 4: The rate of respiratory tract disease in dogs by ages

Age groups	No. of examined (dog)	No. of disease (dog)	Percentage (%)
I (<6 months)	227	39	17.18[a]
II (6 months - 2 years)	173	29	16.76[a]
III (>2 years - 5 years)	175	30	17.14[a]
IV (>5 years)	161	45	27.95[b]
Total	736	143	19.43

[a, b]: Means with different letters in the same column are significantly different (P<0.05)

As shown in Table 4, the rate of respiratory tract disease in dogs by ages was significantly different (P<0.05). The group of over 5 years of age had the highest rate (27.95%), followed by group I (<6 months) (17.18%), group III (>2 years - 5 years) (17.14%) and the lowest rate was found in group II (6 months - 2 years) (16.76%). This result is consistent with Kuehn (1990) and King (2004) that respiratory diseases are common in dogs. Although clinical signs such as coughing and dyspnea are commonly referable to primary problems of the respiratory tract, they may also occur secondary to disorders of other organ systems. Both young and aged animals are at increased risk of developing respiratory disease. At birth, the respiratory and immune systems are incompletely developed; this facilitates the introduction and spread of pathogens within the lungs, and alveolar flooding may occur. In aged animals, chronic degenerative changes that disrupt normal mucociliary clearance and immunological anergy may render the lungs more vulnerable to airborne pathogens and toxic particulates (Kuehn, 1990; King, 2004). Age-related differences should be considered when using the clinical diagnosis in dogs with respiratory tract disease.

3.1.2 The prevalence of respiratory tract disease in dogs by breeds

Result on respiratory tract disease in dogs by breeds was presented in Table 5.

Table 5: The rate of respiratory tract disease in dogs by breeds

Breed groups	No. of examined (dog)	No. of disease (dog)	Percentage (%)
Domestic	346	54	15.61[a]
Foreign	390	89	22.82[b]
Total	736	143	19.43

[a, b]: Means with different letters in the same column are significantly different (P<0.05)

Table 5 showed that the rate of respiratory tract disease in dogs by breeds was significantly different (P<0.05). The ratio of respiratory tract disease in foreign dogs breeds (22.82%) was significantly higher (P<0.05) than that in domestic dog breeds (15.61%). Similar results were verified by Ly Thi

Lien Khai (2017) that who observed that this rate in foreign dog breeds was higher than domestic dog breeds with 12.75% and 7.99%, respectively (P<0.05). Foreign dog breeds are increasingly common, but canine disease has been associated with increased respiratory tract disorders. Predisposition to respiratory tract disorders including stenotic nares, enlarged tonsils, elongated soft palate, everted lateral saccules of the larynx and collapse of the larynx have been reported in foreign dog breeds. Individual dogs may have one or a combination of such respiratory tract conditions which can additionally predispose to other respiratory tract disorders and can be variously combined to describe an overall brachycephalic obstructive airway syndrome (O'Neill *et al.*, 2015). O'Neill *et al.* (2015) observed that upper respiratory tract disorders are relatively commonly diagnosed across Bulldog, French Bulldog, Pug, Border Terrier and Yorkshire Terrier dogs in England. The three evaluated breed types of respiratory tract conformation (Bulldog, French Bulldog and Pug) were relatively short-lived and predisposed to respiratory tract disorders. Breed-related differences should be considered when using the clinical diagnosis in dogs with respiratory tract disease.

3.1.3 The prevalence of respiratory tract disease in dogs by sex

Result on respiratory tract disease in dogs by sex was presented in Table 6.

Table 6: The rate of respiratory tract disease in dogs by sex

Gender groups	No. of examined (dog)	No. of disease (dog)	Percentage (%)
Male	387	82	21.19
Female	349	61	17.48
Total	736	143	19.43

Table 6 showed that the rate of respiratory tract disease in dogs by male sex (21.19%) was higher compared to female sex (17.48%). However, there was no difference (P>0.05) on respiratory tract disease in dogs by sex. A variety of bacteria normally lives in the canine nasal passages, throat, trachea and sometimes lungs without causing signs of illness. Infections by these usually harmless bacteria may occur when the respiratory defense mechanisms are weakened by another infection, irritant or disease. Disease organisms may continue to live in the respiratory tract of recovering animals. When stressed, these animals may relapse; they can also act as a source of infection for other animals. Poor management practices (such as overcrowding) are often associated with poor sanitation and environmental conditions, which can lead to more frequent and more severe infections (Kuehn, 1990; Russell *et al.*, 1991; King, 2004). Similarly, Ly Thi Lien Khai (2017) suggested that the rate of respiratory tract disease in dogs based on gender was no difference (P>0.05).

3.1.4 The prevalence of respiratory tract disease in dogs by rearing modality

Result on respiratory tract disease in dogs by rearing modality was presented in Table 7.

Table 7: The rate of respiratory tract disease in dogs by rearing modality

Rearing modality	No. of examined (dog)	No. of disease (dog)	Percentage (%)
Captive	438	70	15.98[a]
Kept free	298	73	24.50[b]
Total	736	143	19.43

[a, b]: Means with different letters in the same column are significantly different (P<0.05)

As shown in Table 7, the rate of respiratory tract disease of dogs in the free kept groups was significantly different (P<0.05) compared to that with captive group. According to Kuehn (1990) and King (2004), showed that the most important function of the respiratory system is to deliver oxygen into the blood, which distributes it throughout the body and to remove carbon dioxide from the blood. The exchange of oxygen and carbon dioxide occurs in the alveoli. When this exchange fails or becomes inefficient because of disease, the animal can become seriously ill. Large airborne particles usually land on the mucous lining of the nasal passages, larynx, trachea, and bronchi, after which they are carried to the throat to be either swallowed or coughed up. Small particles and microorganisms are destroyed by the body's immune system. In the dogs with kept free group, lung and airway disorders are often caused by direct infection with viruses, bacteria, fungi, parasites, inhalation of irritants or toxic substances. Similar results were verified by Ly Thi Lien Khai (2017) that the rate of respiratory tract disease in dogs based on kept free (13.12%) was higher compared to captive (9.62%), this difference was statistically significant (P<0.05).

3.1.5 The popular clinical symptoms of respiratory tract disease in dogs

Result on the popular clinical symptoms of respiratory tract disease in dogs was presented in Table 8.

Table 8 showed that the rate of popular clinical symptoms of respiratory tract disease in dogs: cough combined nasal discharge at the highest rate (18.18%), followed by cough combined nasal discharge and eye rheum (13.99%), increase of respiratory rhythm (13.29%), cough combined nasal discharge and increase of respiratory rhythm (10.49%), nasal discharge combined eye rheum (10.49%), cough combined nasal discharge and rales (9.79%), cough combined rales and depression (6.99%), dry cough (6.29%), cough combined increase of respiratory rhythm and depression (5.59%), nosebleed (2.80%) and nosebleed combined increase of respiratory rhythm (2.10%). The result shows that the typical clinical manifestations of respiratory tract disease in dogs such as cough, nasal discharge and increase of respiratory rhythm, etc. Lappin *et al.* (2017) stated that upper respiratory tract disease is a syndrome consisting of clinical signs that can include serous to mucopurulent ocular and nasal discharges, epistaxis, sneezing, and conjunctivitis. Pham Ngoc Thach (2008) also reported that the classic signs of canine respiratory tract infection are very similar to the symptoms of the common cold in people. The symptoms of clinical disease in domestic dogs will depend upon the underlying cause of the condition. Dogs with respiratory tract infections typically develop one or more of the following symptoms: sneezing, snorting, coughing (deep, dry and hacking or moist and productive), nasal irritation, nasal discharge, fever (low-grade), difficulty breathing (dyspnea), tiredness, loss of appetite (inappetence), weight loss. Pham Ngoc Thach *et al.* (2012) and Ly Thi Lien Khai (2017) shared the similar results that the typical clinical manifestations of respiratory tract disease in dogs including increase of respiratory rhythm, cough, nasal discharge, dyspnea, rales and depression, etc.

Table 8: The rate of popular clinical symptoms

Clinical symptom	No. (dog)	Percentage (%)
Cough + Nasal discharge	26	18.18
Cough + Nasal discharge + Eye rheum	20	13.99
Increase of respiratory rhythm	19	13.29
Cough + Nasal discharge + Increase of respiratory rhythm	15	10.49
Nasal discharge + Eye rheum	15	10.49
Cough + Nasal discharge + Rales	14	9.79
Cough + Rales + Depression	10	6.99
Dry cough	9	6.29
Cough + Increase of respiratory rhythm + Depression	8	5.59
Nosebleed	4	2.80
Nosebleed + Increase of respiratory rhythm	3	2.10
Total	143	100.00

3.2 Results of the effect of treatments

After 5-7 days of treatment in respiratory tract disease by using antibiotics, most of the clinical symptoms decreased by times. Result on the effect of treatments in respiratory tract disease in dogs was presented in Table 9.

Table 9: The effect of treatments in respiratory tract disease in dogs

Items	Upper airways					Lower airways				
	No. (dog)	Good control		Bad control		No. (dog)	Good control		Bad control	
		No.	(%)	No.	(%)		No.	(%)	No.	(%)
Cef	20	18	90.00	2	10.00	10	9	90.00	1	10.00
Mar	20	19	95.00	1	5.00	10	9	90.00	1	10.00

Cef: Cefuroxime; Mar: Marbofloxacin

The results in Table 9 indicated that treatment by using Marbofloxacin had the good control of upper airways (95.00%) higher compared to Cefuroxime (90.00%); treatment by using Marbofloxacin or Cefuroxime had the same rate in good control of lower airways (90.00%). Marbofloxacin is a synthetic, bactericidal antimicrobial, belonging to the fluoroquinolone group which acts by inhibition of DNA. Marbofloxacin is effective against a wide range of Gram-positive bacteria (in particular *Staphylococci,*

Streptococci) and Gram-negative bacteria (*Escherichia coli, Salmonella typhimurium, Klebsiella spp, Shigella spp, Pasteurella spp, Haemophilus spp, Pseudomonas spp, Brucella canis, Mycoplasma spp)*. Cefuroxime is in a class of medications called cephalosporin antibiotics that works by killing bacteria. Cefuroxime injection is used to treat certain infections caused by bacteria including pneumonia and other lower respiratory tract infections; meningitis and urinary tract infections (Vo Thi Tra An, 2014). Marbofloxacin and Cefuroxime were found as the effective antibiotics for treating respiratory tract disease in dogs. However, the treatment by using Cefuroxime had the bad control of upper airways (10.00%) higher compared to Marbofloxacin (5.00%); using Marbofloxacin and Cefuroxime had the same rate of treatment in the bad control of lower airways (10.00%). The reason is because the owners do not take care of dogs properly, dogs are not kept warm, respiratory tract disease change from chronic to acute respiratory infections.

4 CONCLUSION AND SUGGESTION

The rate of respiratory tract disease in dogs are 19.43% in the Animal Clinic of Can Tho University. The percentage of respiratory tract disease varies among ages, breeds and rearing modality. Foreign dog breeds are more prone to respiratory tract disease than domestic dog breeds. Dogs of over 5 years of age have the highest risk of respiratory tract disease (27.95%). Clinical symptoms include cough, nasal discharge, increase of respiratory rhythm, rales, etc. Marbofloxacin or Cefuroxime could control clinical symptoms in respiratory tract disease in dogs.

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Study on the optimal conditions for fucoidan extraction from *Kappaphycus alvarezii*

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ABSTRACT

Fucoidan, a polysaccharide composed of L-fucose with sulfate ester, has a wide range of pharmacological uses due to its massive number of biological activities. Besides, brown seaweed, fucoidan is also found from Kappaphycus alvarezii alga in the central coast of Vietnam. In the study, the influences of fucoidan extraction from Kappaphycus alvarezii alga by using HCl was assessed. The response surface methodology was applied to optimize three factors of material-to-solvent ratio, temperature, and time for the extraction. The monosaccharide composition was determined by using high-performance liquid chromatography. The fucoidan structures were studied using Fourier transform infrared (FT-IR) and ^{13}C-NMR (nuclear magnetic resonance). Antioxidant activity was determined using DPPH method. Monosaccharides of fucoidan were determined by high-performance liquid chromatography. The optimal results were HCl 1.39M at 85.1℃ in 3.64 hours; fucoidan content was 47.9 µg/mL. The FT-IR spectrum of fucoidan contains specific peaks of sulfate group in fucoidan in the axial or equatorial position. ^{13}C-NMR spectrum and monosaccharides of fucoidan were specific characteristic properties.

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1 INTRODUCTION

Fucoidans, polysaccharides containing substantial percentages of L-fucose and sulfate ester groups, are constituents of seaweeds, with many functions related to the physiological activity (Ale *et al.,* 2011; Fitton, 2011). Fucoidan has many benefits for good health, such as being used for functional foods, anti-cancer, immune, anti-inflammatory, antiviral, anticoagulant and antioxidant (Ale *et al.*, 2001; Kraan, 2012, Lakmal, 2014). Seaweeds have been known as huge fucoidan sources (Hahn *et al*., 2012). There are many methods of pre-treatment, solvents for extraction, precipitate, and anion exchange chromatography to purify fucoidan from the extract. The pretreatment is essential to remove chlorophyll, mannitol, salt, and other small compounds. Methanol–chloroform–water (MeOH-$CHCl_3$-H_2O, 4:2:1) (Ale, 2012) or ethanol 80-85% are usually used as pretreatment solvents (Yang *et al.*, 2008). Acid (Hahn *et al*., 2012) or water (60 − 100°C) (Luo *et al.*, 2009) can also be used as extraction solvents, and $CaCl_2$ is sometimes used to precipitate alginate during the extraction process (Bilan *et al.*, 2002). Extraction with acidic solutions, such as HCl, improved fucoidan yields (Kawamoto *et al.*, 2006). The addition of $CaCl_2$ to precipitate alginate can increase the purity of fucoidan but may also reduce its yield.

Kappaphycus alvarezii, a species of red algae, is one of the most important commercial sources of carrageenans. Carrageenans are grouped according to their sulfation patterns and distributions of 3,6-

anhydro-D-galactose residues into several families of carrageenan sub-types. Fayaz *et al.* (2005) analyzed *Kappaphycus alvarezii* for its chemical composition and found that this species is rich in protein (16.2% w/w), fiber (29.4% w/w) and carbohydrates (27.4% w/w), with a high proportion of unsaturated fatty acids (44.5% of the total; 11.0% oleic acid, 13.5% *cis*-heptadecenoic acid, 2.3% linoleic acid) and saturated fatty acids (37.0%, composed mainly of heptadecanoic acid). The bioactivity of sulfated polysaccharides like carrageenan depends on the degree and position of sulfation, the molecular weight, and the sugar type or glycosidic branching, among other features. The bioactivity of sulfated polysaccharides depends on several structural features such as the degree of sulfation (DS), the molecular weight, the sulfation position, type of sugar, and glycosidic branching. Chemical modification of the carbohydrates can lead to differences in their biological activities (Yuan *et al.*, 2011). Since 1993, *Kappaphycus alvarezii* has become widespread in Vietnam. However, it should be studied more about antioxidant compounds besides carrageenan in order to improve the value of this resource. The aim of this present work is to find suitable conditions for fucoidan extraction from *Kappaphycus alvarezii* on the central coast of Vietnam.

2 MATERIALS AND METHODS

2.1 Materials

Fresh alga *Kappaphycus alvarezii* was collected in Dam Mon area (Khanh Hoa province, Vietnam). Salt, sand, and epiphytes were removed with tap water. The samples were then rinsed carefully with fresh water and stored in a plastic bag at -5°C. To prepare for the experiment, the alga was dried to about 10% moisture, ground and sieved to one mm inhomogeneous size.

Dried alga and salted alga were bought at Dai Hai Foods Company (Ho Chi Minh city). Salted seaweed has rinsed the impurities.

2.2 Methods

2.2.1 Fucoidan extraction protocol

The algal powder was mixed with ethanol 80% and stirred for at least 12 hours at room temperature to remove lipids and pigments. Then the solutions were centrifuged to remove the supernatant. The remaining sediment was then dried at 50°C to remove the remaining alcohol prior to fucoidan extraction with solvent. After centrifugation, TCA (trichloroacetic acid) was added to the supernatant to precipitate protein (4°C, 30 minutes). The solution was then centrifuged, and the supernatant was harvested.

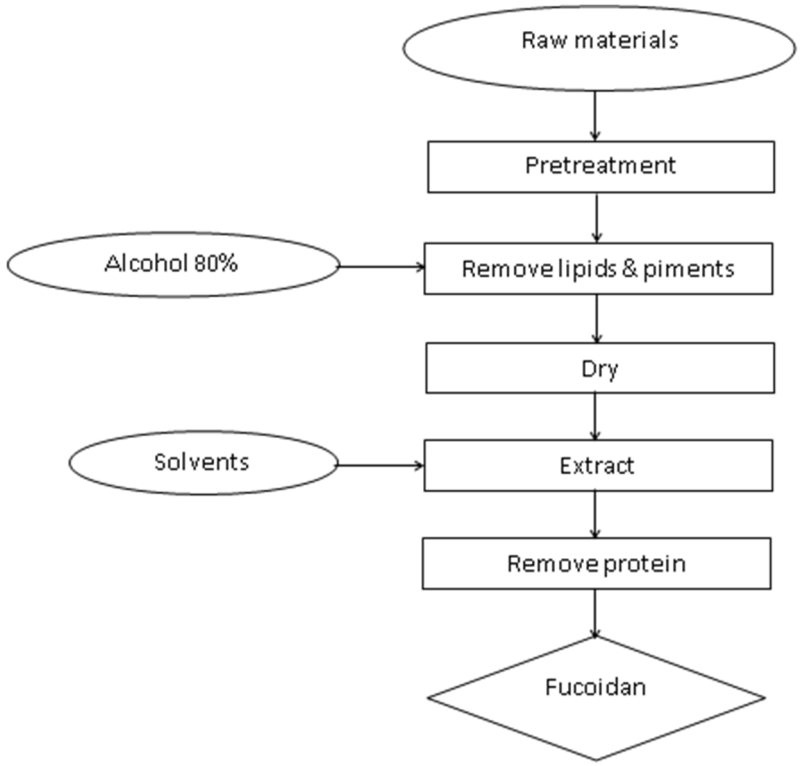

Fig. 1: Fucoidan extraction protocol

2.2.2 Investigation of fucoidan extraction conditions

The initial step of the preliminary experiment was to select appropriate seaweed type of *Kappaphycus alvarezii* alga for fucoidan extraction. Three different seaweed types, namely fresh alga, salted alga, and dry alga were examined. Based on fucoidan content, the best type was chosen. The second step of the preliminary experiment was to determine the medium extraction. Three selected media were 1 M $BaCl_2$, 1 M HCl and distilled water. The next step was to examine the solid-to-solvent ratio at different ratios (1/10, 1/20, 1/30, 1/40, 1/50, and 1/60). The final step of the preliminary experiment was to select the appropriate extraction time course for extraction of fucoidan during various extraction times (1 hour, 2 hours, 3 hours, 4 hours, and 5 hours).

2.2.3 Optimal conditions for fucoidan extraction

Optimization of fucoidan extraction from *Kappaphycus alvarezii* was carried out using the Response Surface Method (RSM). A central composite rotatable design (CCRD), five levels (±α, 0, ±1) consisting of 20 experimental runs were employed including six replicates at the center point.

All the runs were carried out in duplicate. The design variables were the solvent concentration (X1, %), extraction temperature (X2, °C) and the time (X3, min) while the response variable was fucoidan content.

2.3 Analytical methods

2.3.1 Fucoidan determination by spectrophotometer

Standard curve setting: Standard fucoidan (Sigma) was used as the standard. Three replicates of the standard were prepared in different concentrations with distilled water ranging from 10-100 µg/ml. Sample solution of each concentrations (1.0 mL) was added to a standard test tube. The test tubes were cooled on the ice at 4^0C (2-3 minutes), 4.5 mL of sulfuric acid (85%) is added and the samples are homogenized with the help of glass stirrer. Tubes were then cooled under running tap water, and 0.3% cysteine hydrochloric acid was added to the tubes and mix. Tubes were placed in darkness for 2 hours, then the absorbance was measured on a spectrophotometer at 396 nm and 430 nm. A blank with distilled water treated under the same manner was used for the zero. The two absorbance values were subtracted through the following equation: Absorbance = (A396 nm – A427 nm).

Determination of fucoidan content: The same above protocol for fucoidan determination from the samples, in which the sample is replaced by standard fucoidan.

2.3.2 Monosaccharide composition of Fucoidan

Determination of the monosaccharide composition of fucoidan was the first important stage in the investigation of fucoidan structure. The general protocol is known as hydrolysis of fucoidan into monosaccharides before analyzing each monosaccharide by HPLC. Fucoidan sample (5 mg) in tube 5 mL, was added 1mL TFA (trifluoroacetic acid) 2 M and shaken. Fucoidan was hydrolyzed at 100^0C in 6 hours. Then they were vacuum evaporated prior washed three times with MeOH to remove remained TFA. The resin was dissolved with 1 mL deionized water to the solution. This solution was used to analyze monosaccharides on the machine of IC-500 Biotrongik (Germany), Shim-pack ISA- 07/S2504 column (0.4 x 25 cm), a mobile phase of borat kali buffer, the flow rate at 0.6 mL/min. Monosaccharides were analyzed by HPLC. The purified form of fucose, glucose, galactose, mannose, xylose, rhamnose were used as standards.

2.3.3 Determination of fucoidan spectra by the IR method

Objectives: The specific peak of sulfate in fucoidan was determined by infrared spectra (IR).

The FT-IR infrared spectra of fucoidan were recorded by the machine of Tensor 37 Brucker (at the Center for Critical Analysis, Ho Chi Minh City University of Technology) with KBr beam detector in an absorbance mode 400-4000 cm^{-1}. The trembling was recorded as a graphic representation.

2.3.4 Determination of fucoidan structure by NMR spectra

The application of nuclear magnetic resonance (NMR) spectroscopy would also be very useful to obtain more structural information on fucoidan by identifying the present residues and how this polysaccharide is linked together. The side chains can also be determined and should lead to a much better understanding of the various biological properties that fucoidan have. The spectra were obtained on a spectrometer provided with a 5 mm probe at room temperature. The solution of polysaccharide samples in H_2O was sonicated at 20 kHz, and then D_2O was added to produce a solution containing calcium 40 mg in 0.4 mL of 1:1 H_2O – D_2O. Acetone was added as internal standard

(referred to Me_4Si by calibrating the acetone methyl group to 31.1 ppm). Typical parameters were as follows: maximum acquisition time, no relaxation delay, 90°-pulse angle, and 40,000 scans.

2.4 Data analysis

Statistically significant differences between samples were analyzed using a one-way analysis of variance (ANOVA) test with Microsoft Excel 2013 and IBM SPSS Statistics 20 software. P values less than 0.05 were considered statistically significant. Optimal data were handled by using JMP 10 software.

3 RESULTS AND DISCUSSION

3.1 The effects of material types and moisture on fucoidan extraction

The effects of types and moisture of materials on fucoidan extraction are shown in Table 1. The amount of fucoidan extracted from fresh alga (26.7 µg/mL) was higher than that in salted alga (19.7 µg/mL) and dry alga (12.8 µg/mL). Thus, fresh alga was selected as the raw material for fucoidan extraction. In addition, at different moistures of 82.5% (fresh alga), 47.3% (medium moisture) and 9.8% (dried alga), the value of fucoidan content obtained was not significantly different (Table 1b). In fresh seaweeds, various microorganisms can decompose plant structure, alginate, and polysaccharide compounds. The drying process can reduce the material moisture, it is facilitated for the storage. In addition, the fucoidan content in algae is dependent on harvest time (Park *et al.*, 1997; Usov *et al.*, 2001). In this study, overmuch fresh alga was harvested, then dried and stored for all experiments.

Table 1a: The effects of material types

Material types	Fucoidan content (µg/mL)
Fresh alga	26.7 ± 0.48^{a}
Salted alga	19.7 ± 0.47^{b}
Dried alga	12.8 ± 0.40^{c}

The numbers with different superscript letters in the same column were significantly different by LSD test (P < 0.05).

Table 1b: The effects of moisture

Material moisture	Fucoidan content (µg/mL)
82.5%	26.7 ± 0.48^{ns}
47.3%	26.4 ± 0.56^{ns}
9.8%	27.2 ± 0.35^{ns}

ns: not significant (P < 0.05).

3.2 The effects of solvents on fucoidan extraction

The appropriate medium extraction ensures fucoidan extracted without denaturation. According to earlier studies (Ponce *et al.,* 2003; Synytsya *et al.,* 2010; Sethi, 2012; Wang and Chen, 2016), 1 M HCl, 1 M $BaCl_2$ and distilled water were chosen to examine the extraction. The results were shown in Figure 2.

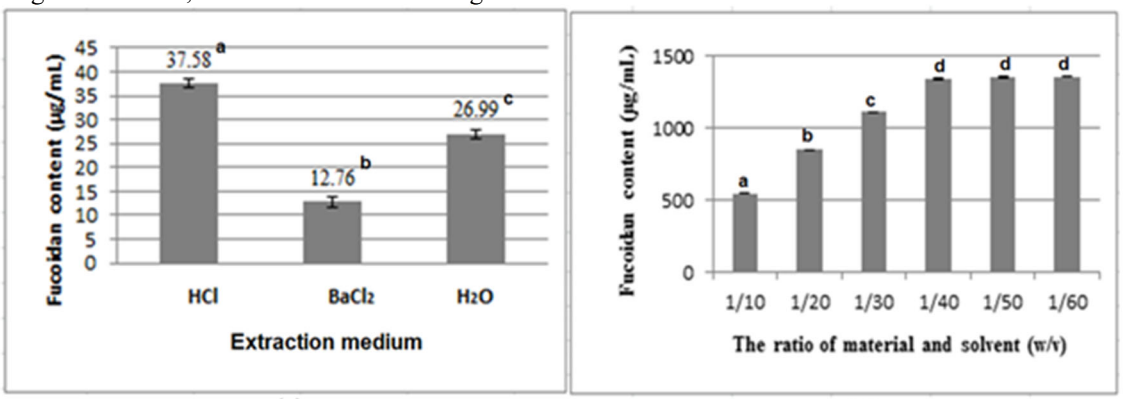

Fig. 2: The effects of solvents (a), solvent ratio (b) on fucoidan extraction

The numbers with different superscript letters in the same column were significantly different by LSD test (P < 0.05).

The yield of fucoidan extraction with 1 M HCl was higher than that of distilled water and $BaCl_2$. HCl is a proper solvent to extract fucoidan in *Kappaphycus alvarezii* algae (Fig. 2a). Besides, fucoidan extraction in HCl lower concentration is more stable than other solvents in case of structure guarantee. Also, using water at high temperature, carrageenan is extracted to increase a viscosity that impedes further stages. Thus, HCl was chosen as the extraction solvent for the following experiments. This result is similar to the published procedure by Black *et al*. 1952.

Figure 2b showed that the value of fucoidan content increased with the increase of solvent volume at the

initial stage. This is due to the fact that higher solvent volume resulted in increasing the capacity extraction of fucoidan. Higher volume of solvent used resulted in a higher amount of fucoidan (the ratio of solvent and material in the range of 1/10, 1/20, 1/30, 1/40). It helps the solvent to penetrate into the material and extract soluble constituents because of the increase of concentration gradient. However, it is not significant at the certain limit which leads to the cost of the solvent and unwanted compounds extracted like carrageenan. It is a cause of viscosity increase and obstacle for later stages. Thus, the suitable ratio of material and solvent was 1/40 (w/v).

3.3 The effects of temperature and time on fucoidan extraction

The effects of temperature and time extraction on the fucoidan content were shown in Figure 3.

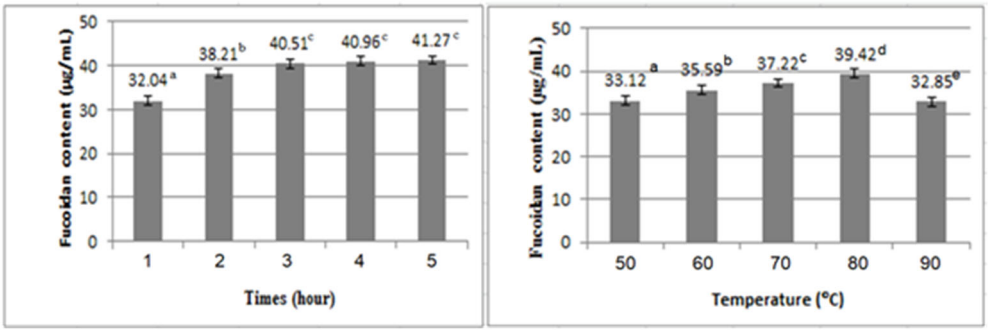

Fig. 3: The effects of temperature (a) and time extraction (b) on fucoidan content

The numbers with different superscript letters in the same column were significantly different by LSD test (P < 0.05).

The results showed that the highest fucoidan content was recorded at 80°C (39.4 µg/mL) (Fig. 3a). In the case of temperature rise, it accelerates the extraction stage, and more fucoidan is extracted. However, at the higher temperature (90°C), carrageenan, agar in algae begins to be extracted (Dunstan *et al.*, 2001; May, 2002). The high viscosity of these compounds inhibits the extraction of fucoidan. Moreover, high temperature leads to degradation in the chemical structure of ingredients.

As shown in Figure 3b, the amount of fucoidan increased over time. However, after three hours, the increase was not significant because the fucoidan content does not increase despite longer time extraction at the threshold limit yield. It also released lots of unwanted compounds (such as alginate, agar), which impede filtration, waste energy and time. Therefore, 80°C and time extraction three hours were chosen for fucoidan extraction from *Kappaphycus alvarezii* by HCl solvent.

3.4 Optimizing fucoidan extraction

Thanks to the above single factor investigation, it was found that solvent concentration, time and temperature extraction had significant effects on fucoidan extraction yield. The results of optimizing three above factors of X_1 (solvent concentration, %), X_2 (temperature, °C), X_3 (time, minute) with a central composite rotatable design CCRD were shown in Table 2.

Table 2: Results of optimizing fucoidan extraction

No.	Factors			Fucoidan content (µg/mL)	No.	Factors*			Fucoidan content (µg/mL)
	X_1	X_2	X_3			X_1	X_2	X_3	
1	-1	-1	-1	25.61	11	0	-1.68	0	35.43
2	-1	-1	1	30.21	12	0	1.68	0	42.47
3	-1	1	-1	35.67	13	0	0	-1.68	36.78
4	-1	1	1	38.09	14	0	0	1.68	42.56
5	1	-1	-1	35.83	15	0	0	0	46.23
6	1	-1	1	38.71	16	0	0	0	45.41
7	1	1	-1	37.42	17	0	0	0	42.35
8	1	1	1	49.19	18	0	0	0	47.28
9	-1.68	0	0	40.46	19	0	0	0	42.46
10	1.68	0	0	43.57	20	0	0	0	47.62

**Factors: X1 (solvent concentration), X2 (temperature), X3 (time)*

The adequacy of the model was checked accounting for R^2 and adjusted-R^2 (Caporaso, 2016). RSM models with correlation coefficient R^2 values higher than 0.80 are considered as valid ones (Joglekar *et al*., 1987). The parameter lack-of-fit is an indication of the adequacy of a model to describe the experimental factors and the response variable, considering the data not included in the regression or some variations that cannot be accounted for random error. The lack of fit illustrated in Table 3 did not result in a significant p-value for the studied variables, therefore meaning that these models were sufficiently accurate for predicting the relevant responses. The regression equation demonstrates the relationship between the fucoidan (Y) content and the influencing factors as follows:

$$Y = 45.33 + 2.69X_1 + 3.06X_2 + 2.30X_3 - 1.89X_1^2 - 2.97X_2^2 - 2.72X_3^2 \quad (Eq.1)$$

Table 3: ANOVA analysis

Source	Degrees of freedom	Sum of squares	Mean square	F value	P value > F
Regression					
Model	9	558.18	62.02	5.029	0.0094*
Error	10	123.32	12.33		
C. Total	19	681.507			
Lack of fit	5	97.39	19.47	3.76	0.863
Pure error	5	25.92	5.18		
Total error	10	123.32			

**Significant at 5% (Fischer's least significance difference test)*

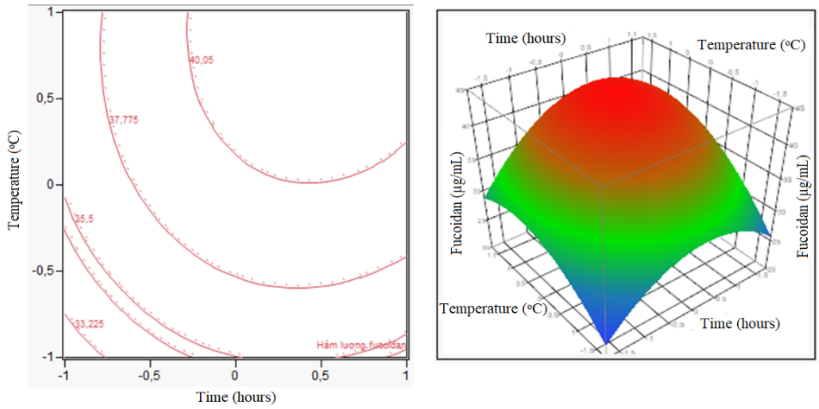

Fig. 4: The response surface and contour plots for the effects of temperature and time extraction on fucoidan content

The non-significant value of lack of fit (F = 3.76) showed that the model is fitted with a good prediction (R^2 = 0.819) (Table 3). The non-significant value of lack of fit (F = 3.28) showed that the model is fitted with good prediction (R^2 = 0.819) (Table 3). Various response 3D surface graphs were generated for fucoidan content and shown in Figure 4. The interaction effect of solvent concentration (X1), extraction temperature (X2) and time (X3) showed significant ($p < 0.01$) positive effect on fucoidan content (Eq. 1). The quadratic effect of variables (X_1^2, X_2^2, X_3^2) significantly affected fucoidan extraction. The software of JMP 10 was used to determine the optimal conditions of fucoidan extraction: X_1 = 0.77 (1.39 M), X_2 = 0.51 (85.1°C), X_3 = 0.64 (3.64 hours). At the optimal extraction conditions, fucoidan content was 47.9 µg/mL.

Three experiments were carried at optimal conditions. Experimental results showed the highest fucoidan content (45.5 µg/mL) when the extraction process was at optimal conditions, this result was consistent with the predictive model (47.9 µg/mL).

3.5 Fucoidan spectra

3.5.1 FT-IR fucoidan spectra

The region around 800–850 cm^{-1} was used to infer the position of the sulfate groups in sulfated polysaccharides. The FT-IR spectroscopy showed typical absorption bands of sulfated polysaccharides (Ribeiro *et al.,* 1994). The FT-IR spectrum of crude fucoidan from *Kappaphycus alvarezii* (Fig. 5) contained broad peak of 800-1732 cm^{-1} (sulfate at axial or equatorial positions), 1028-1637cm^{-1} (C-O-C and C-O-H stretching) (Shanthi *et al*., 2014), 1210-1250

cm^{-1} (S=O stretching) and 840-848 cm^{-1} (C-O-S stretching), which is a characteristic band for deoxysugars as fucose. In addition, crude fucoidan from *Kappaphycus alvarezii* has the characteristic wide band at 1218 cm^{-1} (S=O stretching), the peak of 846 cm^{-1} (C-O-S stretching at C4 of α-L-fucopyranose). The infrared spectrum data only indicates that the sulfate group is in the axial (C4) or equatorial (C2 or C3) of the pyranose ring of fucose or galactose (Bilan *et al*., 2004; Rodriguez-Jasso *et al.*, 2011; Yuan *et al.*, 2015).

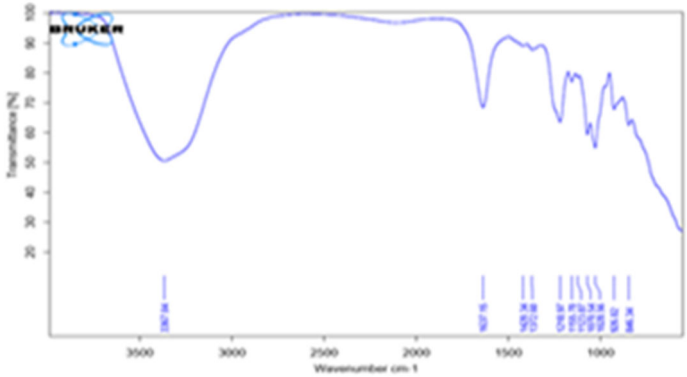

Fig. 5: FT-IR fucoidan spectra

The FT-IR was typical of fucoidans in the range of 1240-1255cm^{-1} (Marais M.F and Joseleau J.P, 2001; Rodriguez-Jasso *et al*., 2011). Rodriguez-Jasso (2013) also suggested that the typical IR absorption spectrum of fucoidan from 1240 to 1255cm^{-1} is characterized by S = O bond sulfate in polysaccharides (Rodríguez-Jasso *et al*., 2013). The absorption band at 1220-1230 cm^{-1} for S = O, 840 cm^{-1} for C4 axial, 820 cm^{-1} for C-O-S at equatorial C2 and C3 of sulfate (Yuan *et al.*, 2015). Zayed *et al*., 2016 reported the FT-IR spectrum of fucoidan_M is defined and explained: a peak at 1218 cm^{-1} (S = O stretching), 1005 cm^{-1} (C-O ether), 835 cm^{-1} (C-O-S stretching) of sulfate. Fucoidan infrared spectra at peaks: 3200-3550 cm^{-1} (strong and pointed) for O-H, 1020-1080 cm^{-1} (mean and sharp) for C-O glycoside, 1240-1260 cm^{-1} (mean) for S = O of sulfate. In addition, the two peaks at 820 and 840 cm^{-1} were determined for O-S ester sulfate, where the equatorial sulfate and axial ester are characterized at the peak of 820 cm^{-1} and 840 cm^{-1}, respectively. These peaks are characterized by fucoidan (Thi *et al.,* 2012).

3.5.2 ^{13}C-NMR spectroscopy

The ^{13}C-NMR spectra of fraction 2 are showed in Figure 6.

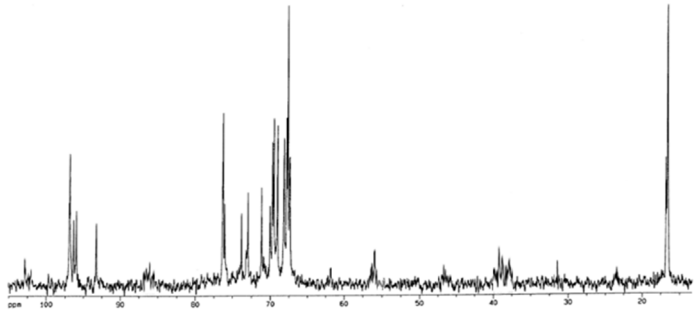

Fig. 6: ^{13}C-NMR spectra of fucoidan from *Kappaphycus alvarezii*

Like many other algal fucoidans, sulfated polysaccharides from *Kappaphycus alvarezii* had very complex ^{13}C-NMR spectrum, which was difficult to interpret completely (Figure 6). The ^{13}CNMR spectrum of fucoidans exhibited several major signals, most of them showing a certain degree of multiplicity. The multiplicity of signals with slightly different chemical shifts suggested either diversity in the positions of glycosidic bonds, or/and multiplicity of the patterns of sulfation in the fucose residues. ^{13}C-NMR spectrum of fucoidan contained several intense signals in the anomeric (97-102 ppm) and high-field (15-18 ppm) regions, which are typical of a-L-fucopyranose residues, the signal anomeric 67-86 ppm is C2-C5 carbon of the pyranoid ring (Bilan

et al., 2002). In addition, the ^{13}C-NMR spectrum also shows the presence of β-D-Galactose through the signal of the non-bonding C6 and C1 of the β-D-Galactose, corresponding to signal in the anomeric 61-62 ppm and 103-104 ppm (Vishchuk *et al.,* 2011). Thus, the ^{13}C-NMR spectrum depicted that the fucoidan samples had the structural characteristics belonged to the sulfated carrageenan group.

3.6 Monosaccharide determination

Monosaccharide composition in fucoidan such as fucose, mannose, galactose, xylose, and glucose was determined by HPLC. The content of D-xylose in fucoidan, sulfate and uronic acid was obtained (Table 4).

Table 4: Composition of monosaccharide in fucoidan

Fucoidan fractions	SO_4^{2-}	Acid uronic	Monosaccharide composition				
	(%)		%				
			Fucose	Mannose	Galactose	Xylose	Glucose
F-F1	15.67	10.5	18.34	11.17	17.87	8.4	6.5

Table 4 showed that fucose in fucoidan from *Kappaphycus alvarezii* occupies a higher content (18.34%) than other monosaccharides. Galactose content was 17.87%, which was equivalent to fucose. In addition, there were also other sugars with a lower content as mannose (11.17%), xylose (8.4%), and glucose (6.5%). Sulfate content and its position on the sugar were the most important factors, influencing the biological activity of fucoidan. The content of sulfate and uronic acid in fucoidan from *Kappaphycus alvarezii* was 15.67% and 10.5%, respectively. These results were also consistent with previous publications on the diversity of the chemical composition of fucoidan. Fucose had a significant content in fucoidan (35.8-55.85%), galactose was equal to fucose, mannose (2.5-19.2%), xylose (1.3-11.5%) and glucose (0-20.6%). This showed that monosaccharide content in fucoidan from *Kappaphycus alvarezii* is a remarkable difference with fucoidan from other seaweeds. It was lower fucose and higher uric acid content. This inferred the diversity of the chemical composition of fucoidan in different species.

4 CONCLUSIONS

For fucoidan extraction from *K. alvarezii,* HCl is a suitable solvent, the ratio of material/solvent 1/40 (w/v), the temperature of 80°C in 3 hours. The optimal condition for fucoidan extraction is 1.39 M HCl, temperature 85.1°C, 3.64 hours (CCRD experiment design), fucoidan content of 47.9 µg/mL. FT-IR, ^{13}C-NMR spectrum and monosaccharides referred the sulfated ester absorption from *Kappaphycus alvarezii* are characterized for fucoidan.

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Chemical compositions, bioactive compounds, and physicochemical properties of different purple sweet potato flours

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ABSTRACT

The difference in growing conditions, cultivating techniques or genotypes resulted in various quality of purple sweet potatoes. The objective of this study was to determine chemical compositions, bioactive compounds (total anthocyanin, phenolic and flavonoid contents) and physicochemical properties (swelling and water solubility indexes, and pasting properties) of flours prepared from purple sweet potatoes grown at five villages in Dong Thap and Vinh Long provinces. In term of dry basis, the chemical compositions of different purple sweet potato flours consisted of 1.08-3.09% of protein, 0.17-0.41% of lipid, 2.49-2.78% of ash, and 93.94-95.92% of total carbohydrate. Purple sweet potatoes grown at Phu Long village (Chau Thanh district, Dong Thap province) had the highest total anthocyanin content (6.8 mg cyanidin-3-glucoside/100 g flour), total phenolic content (202.2 mg FAE/100 g flour), total flavonoid content (85.6 mg RE/100 g flour) and water solubility index (13.6%) as compared to other purple sweet potato flours. Nevertheless, the paste of purple sweet potato from Hoa Tan village (Chau Thanh district, Dong Thap province) had the highest swelling index (7.5 g water/g flour) and manifested the greatest resistance against retrogradation, gel consistency and hot paste stability among other flours. The results of this study provided the useful information about the quality of purple sweet potatoes grown at different locations.

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1 INTRODUCTION

Sweet potato (*Ipomoea batatas* L.) is considered as one of the most important tubers in term of annual production in over the world (Sharma and Kaushal, 2016) and nutritional values. It contains a great amount of carbohydrate, dietary fiber, minerals, vitamins, and bioactive compounds like phenolic acids and anthocyanin. Sweet potatoes may be classified based on flesh color that ranges from white to yellow, orange, and deep purple (Onwueme, 1978). Purple sweet potatoes with two specified crops like winter-spring and summer-autumn have been extensively grown at Dong Thap, Vinh Long and Kien Giang provinces (Mekong Delta, Vietnam).

Phenolic compounds are one group of major antioxidants of fruits and vegetables, including sweet potato (Teow *et al.*, 2007). They play an

important role in a therapeutic treatment and as the preventive potential of several chronic diseases including cardiovascular disease and possibly certain cancers (Scalbert *et al.*, 2005). Preceding researches proved that phenolic compounds found in sweet potato were helpful in restricting the growth of human colon, leukemia and stomach cancer cells (Kurata *et al.*, 2007), and alleviating diabetes in humans (Ludvik *et al.*, 2008).

Anthocyanin, one of the primary water-soluble pigments, is responsible for colors of fruits and plants ranging from blue to red (Gross, 1987). It could be found widely in some plant families, like *Vitaceae*, *Rosaceae*, *Solanaceae*, *Saxifragaceae*, *Cruciferae* and *Ericaceae* (Jackman and Smith, 1996). Grapes, red cabbages, blood oranges and fruit berries are considered as the best sources of anthocyanin, while black carrots, and purple sweet potatoes contain a minority (Mateus and de Freitas, 2008). Previous studies revealed that anthocyanin plays an important role in performing anti-inflammatory function, inhibiting the growth of microbes, protecting nerve system, and lowering the risk of carcinogenic and cardiovascular diseases (Oki *et al.*, 2002; Wallace, 2011).

In this research, chemical compositions, bioactive compounds (total flavonoid compounds, total anthocyanin content, and total phenolic compounds), and physicochemical properties (swelling index, water solubility index, and pasting properties) of five purple sweet potato flours (PSPF) (Hoa Tan, Tan Phu, Phu Long, Tan Thanh, and Thanh Dong) were investigated.

2 MATERIALS AND METHODS

2.1 Materials

A purple sweet potato (*Ipomoea batatas* L.), popularly grown at Hoa Tan, Tan Phu, and Phu Long villages (Chau Thanh district, Dong Thap province), and Tan Thanh and Thanh Dong villages (Binh Tan district, Vinh Long province), was used in this study. The vines were planted in July, 2017 and harvested in October, 2017. All tubers were in the uniformity of shape and size (200 – 300 g/tuber), and were examined for the absence of insect contamination like *Cylas formicarius*. PSPF prepared from tubers grown at Hoa Tan, Tan Phu, Phu Long, Tan Thanh and Thanh Dong villages were coded as HT, TP, PL, TT, and TD, respectively.

Ferulic acid, Rutin, and Folin-Ciocalteu reagent were purchased from Sigma Co. Ltd. Other chemicals were bought from chemical store in District 10, Ho Chi Minh City.

2.2 Methods

2.2.1 Flour preparation

Fresh tubers were carefully washed with water to remove all contaminants and drained at room temperature (28 ± 2^{o}C). Then they were peeled, sliced into pieces (3 cm), and then dried in the convection oven at 55^{o}C until moisture content of 10-11% (approximately 24 hours). Then, fine flour was obtained by grinding with analytical mill (A11 Basic, IKA, Germany).

2.2.2 Extraction method

Ethanol-assisted extraction, as a modified method of Tran Ngoc Tan and Pham Van Hung (2014), was applied to obtained extract from purple sweet potato flour. Flour (2.5 g) and 50 mL of 25% ethanol were mixed in a flask and then shaken in an incubator at 150 rpm for 2 hours. After centrifuging at 5,000 rpm for 15 min, the supernatant was collected and evaporated at 50^{o}C to obtain residue. Then, it was reconstituted with distilled water to a final volume of 50 mL, and stored at 4^{o}C until it was used.

2.2.3 Determination of chemical compositions of purple sweet potato flours

The AACC (American Association of Cereal Chemists) approved methods 46-10.01, 30-10.01, and 08-01.01 (AACC, 2000) were used to analyze protein, lipid, and ash contents of PSPF, respectively. Total carbohydrate content was calculated from the subtraction of protein, lipid and ash contents.

2.2.4 Determination of bioactive compounds of purple sweet potato flours

Total phenolic contents (TPC) in the extract of PSPF were measured using spectrophotometric methods as previously described by Liyana-Pathirana and Shahidi (2006) with minor modification. The mixture of 0.5 mL of extract and 0.5 mL of Folin-Ciocalteu reagent was added with 1 mL of saturated sodium carbonate solution, and followed by adjusting the volume to 10 mL distilled water. After mixing well, the whole mixture was stabilized at room temperature for 45 min in the dark, followed by centrifuging at 5,000 rpm for 10 min. After that, the absorbance of clear supernatants from samples or standard solution prepared from ferulic acid was recorded at 725 nm. TPC was calculated and expressed in micrograms of ferulic acid equivalent per 100 grams of sample in dry basis (mg FAE/ g flour, dry basic [db]).

Total flavonoid contents (TFC) in the extract of PSPF were evaluated by a slightly modified method of Al-Farsi and Lee (2008). The mixture of 0.5 mL

of extract and 1.5 mL of ethanol 95% was mixed with 0.1 mL of aluminum chloride solution 10%, and followed by adding 0.1 mL of potassium acetate 1 M, and then 2.8 mL of distilled water. After stabilizing at room temperature for 30 min, the absorbance of samples or standard solution prepared from rutin was measured at 415 nm. TFC was calculated and expressed in micrograms of rutin equivalent per 100 grams of sample in dry basis (mg RE/ 100 g flour, db).

Anthocyanin content in the extract of PSPF was analyzed according to the method reported by Lee *et al.* (2005). Half of milliliter of each extract was prepared in two distinct test tubes, in which one was mixed with 2 mL of potassium chloride buffer (pH 1.0) and other was mixed with 2 mL of sodium acetate buffer (pH 4.5). After mixing and stabilizing at room temperature in the dark for 30 min, the absorbance of each mixture was taken at 520 nm and 700 nm. The amount of anthocyanin was expressed as mg cyanidin-3-glucoside equivalents per liters and calculated by the following formula:

$$Cyanidin - 3 - glucoside = (A\ x\ MW\ x\ DF\ x\ 1{,}000)/(\varepsilon L)$$

Where: $A = (A_{520nm} - A_{700nm})_{pH\ 1.0} - (A_{520nm} - A_{700nm})_{pH\ 4.5}$

MW is molecular weight of cyanidin-3-glucoside (449.2 g/mol)

DF is dilution factor

ε is molar extinction coefficient of cyanidin-3-glucoside (26,900 $L.mol^{-1}.cm^{-1}$)

L is the cell path length (cm)

2.2.5 Determination of physicochemical properties of purple sweet potato flours

Water solubility index (WSI) of PSPF was determined according the method of Kusumayanti *et al.* (2015) with a slight modification. The flour (0.5 g) was dispersed in 10 mL of distilled water, and mixed for 1 min. Then, the mixture was heated in a shaking water bath (200 rpm, 30 min, 90 ^oC). Following this, the cooked paste was rapidly cooled to room temperature, and then centrifuged at 1,600 rpm for 10 min. The supernatant was collected, dried at 120 ^oC for 4 hours, and then weighed. WSI was calculated as the mass of flours dissolved in the supernatant divided by the initial mass of sample and expressed in percentages.

Swelling index (SI) of PSPF was analyzed using the method of Abu *et al.* (2005). One gram of flour was dispersed in 20 mL water and vortexed for 1 min. The tube was heated in a shaking water bath (200 rpm, 30 min, 90 ^oC), then cooled under running tap water, and finally placed in ice water bath for 10 min to accelerate gel formation. After centrifuging (4500 rpm, 10 min, 20 ^oC), the falcon was placed at room temperature for 5 min. The supernatant was carefully removed and the weight of the residue was noted. SI was calculated as the ratio of final residue weight to initial sample weight.

Pasting properties of PSPF were measured using a micro visco-amylo-graph (Brabender® GmbH & Co. KG, Germany). The flour suspension (15%, w/v) was preheated to 30 ^oC, heated up to 93 ^oC at a constant rate of 7.5 ^oC/min and then held at 93 ^oC for 15 min. Then, the paste was cooled to 30 ^oC at the same rate and then held at 30 ^oC for 15 min. The pasting properties of the slurry were recorded as the visco-amylo-graph program described as gelatinization temperature, maximum viscosity, trough viscosity, final viscosity, breakdown and setback.

2.2.6 Statistical analysis

All the experiments were performed at least in triplicate. Analysis of variance (ANOVA) was performed using the Tukey's test with significance level at $p < 0.05$ to compare the means of the results of chemical compositions, bioactive compounds and physicochemical characteristics of PSPF.

3 RESULTS AND DISCUSSIONS

3.1 Chemical compositions of purple sweet potato flours

Proximate compositions of five PSPF are shown in Table 1. The chemical compositions of different PSPF consisted of 1.08 – 3.09% of protein, 0.17 – 0.41% of lipid, 2.49 – 2.78% of ash, and 93.94 – 95.92% of total carbohydrate. Among five purple sweet potato flour samples, PL had the highest protein content (3.09%), TP accounted for the greatest percentage of lipid content (0.41%), while TT contained the highest ash and total carbohydrate contents (2.78 and 95.92%, respectively). These results were consistent with the data revealed by Woolfe (1992) found that chemical compositions of sweet potato consisted of 1.2 – 10.0% of protein, 1.0 – 2.5% of lipid, 0.6 – 4.5% of ash, and 83.0 – 97.2% of total carbohydrate. The results indicated that the chemical composition of purple sweet potato was affected by the growing locations.

Table 1: Chemical compositions of five purple sweet potato flours (%, db)

Flour samples	Protein	Ash	Crude fat	Carbohydrates
HT	2.20 ± 0.06^{b}	2.61 ± 0.01^{ab}	0.17 ± 0.01^{a}	95.03 ± 0.06^{bc}
TP	2.51 ± 0.51^{bc}	2.49 ± 0.18^{a}	0.41 ± 0.03^{d}	94.60 ± 0.69^{ab}
PL	3.09 ± 0.02^{c}	2.66 ± 0.05^{ab}	0.32 ± 0.01^{c}	93.94 ± 0.06^{a}
TT	1.08 ± 0.03^{a}	2.78 ± 0.04^{b}	0.22 ± 0.01^{b}	95.92 ± 0.03^{cd}
TD	1.31 ± 0.06^{a}	2.76 ± 0.02^{b}	0.20 ± 0.01^{ab}	95.73 ± 0.08^{d}

HT: Hoa Tan; TP: Tan Phu; PL: Phu Long; TT: Tan Thanh; TD: Thanh Dong.

Data followed by the same superscript letter in the same column are not significantly different (P <0.05).

3.2 Bioactive compounds of purple sweet potato flours

Table 2 demonstrates the amounts of total phenolic compounds in the extracts of five purple sweet potato flour samples. TPC of PSPF ranged from 173 to 202 mg FAE/100 g flour in dry basis. Among them, PL had the highest amount of total phenolic compounds (202 mg FAE/100 g flour, db), followed by TT with 191 mg FAE/100 g flour (db), while there was no remarkable difference observed among HT, TP, and TD. Previous studies revealed that the amounts of TPC in Taiwanese and Filipino purple sweet potatoes were 6.4 and 434.3-736.8 mg gallic acid equivalent per 100 g flour, respectively (Huang *et al.*, 2006; Rumbaoa *et al.*, 2009).

Table 2: Bioactive compounds (TPC, TFC, and AC) of purple sweet potato flours

Flour samples	TPC (mg FAE/100 g flour, db)	TFC (mg RE/100 g flour, db)	AC (mg cyanidin-3-glucoside/100 g flour, db)
HT	173 ± 10^{a}	73.8 ± 1.5^{b}	3.37 ± 0.07^{a}
TP	185 ± 3^{a}	77.3 ± 3.5^{b}	5.20 ± 0.52^{b}
PL	202 ± 1^{c}	85.6 ± 3.2^{c}	6.83 ± 0.14^{c}
TT	191 ± 1^{b}	78.5 ± 0.8^{b}	3.11 ± 0.35^{a}
TD	183 ± 0^{a}	66.1 ± 1.0^{a}	2.91 ± 0.20^{a}

HT: Hoa Tan; TP: Tan Phu; PL: Phu Long; TT: Tan Thanh; TD: Thanh Dong.

TPC: total phenolic content; TFC, total flavonoid content; AC anthocyanin content.

Data followed by the same superscript letter in the same column are not significantly different (P <0.05).

The amounts of total flavonoid and anthocyanin compounds in the extracts of five purple sweet potato flour samples are also given in Table 2. TFC of these PSPF was in a range of 66.1 to 85.6 mg RE/100 g flour (db) and anthocyanin content of these PSPF was in a range of 2.91 to 6.83 mg of cyanidin-3-glucoside/100 g flour (db). Among them, PL had the highest amount of total flavonoid compounds (85.6 mg RE/100 g flour, db) and anthocyanin (6.83 mg of cyanidin-3-glucoside/100 g flour, db). The TFC of HT, TP, and TT flours were not significantly different, while the lowest TFC belonged to TD (66.1 mg RE/100 g flour, db). The AC of TP was 5.20 mg of cyanidin-3-glucoside/100 g flour (db), significantly higher than HT, TT, and TD flours. These data corresponded with the result reported by Huang *et al.* (2006) who concluded that the amounts of total flavonoid compounds and anthocyanin in Taiwanese purple sweet potato were 34.8 mg gallic acid equivalent per 100 g flour and in a range of 0.5 to 9.0 mg cyanidin-3-glucoside per 100 g flour, respectively. Other studies revealed that anthocyanin content in purple sweet potato flour was around 6.2 mg peonidin-3-glucoside per 100 g flour (Ji *et al.*, 2015). Thus, the amounts of bioactive compounds were found to be significantly different depending on the growing locations.

3.3 Physicochemical properties of purple sweet potato flours

Swelling index (SI) of five purple sweet potato flour samples is illustrated in Figure 1. Among these flour samples, HT had the highest swelling index (7.5 g water/g flour), while the lowest statistics belonged to TD (5.3 g water/g flour). SI of TT, PL and TP was in the line between that of TD and HT. These differences in these SI were mainly due to the presence of lipid (Leach *et al.*, 1959) or amylose content and its properties like amylose-lipid complexation or total leached amylose in addition to phosphate content (Zuluaga *et al.*, 2007).

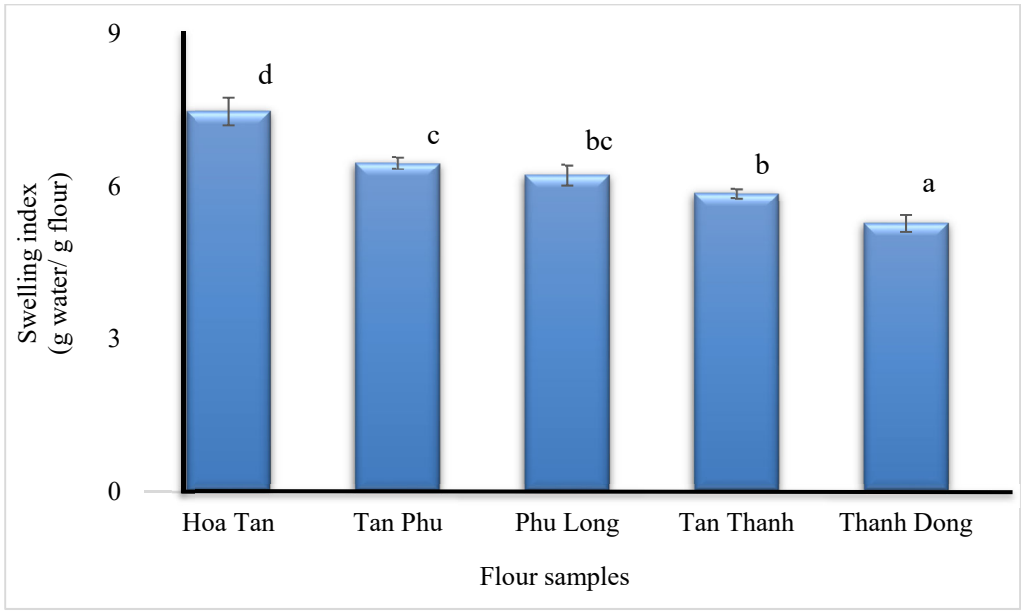

Fig. 1: Swelling index of purple sweet potato flours (g water/ g flour)

Bars followed by the same superscript letter are not significantly different (P <0.05).

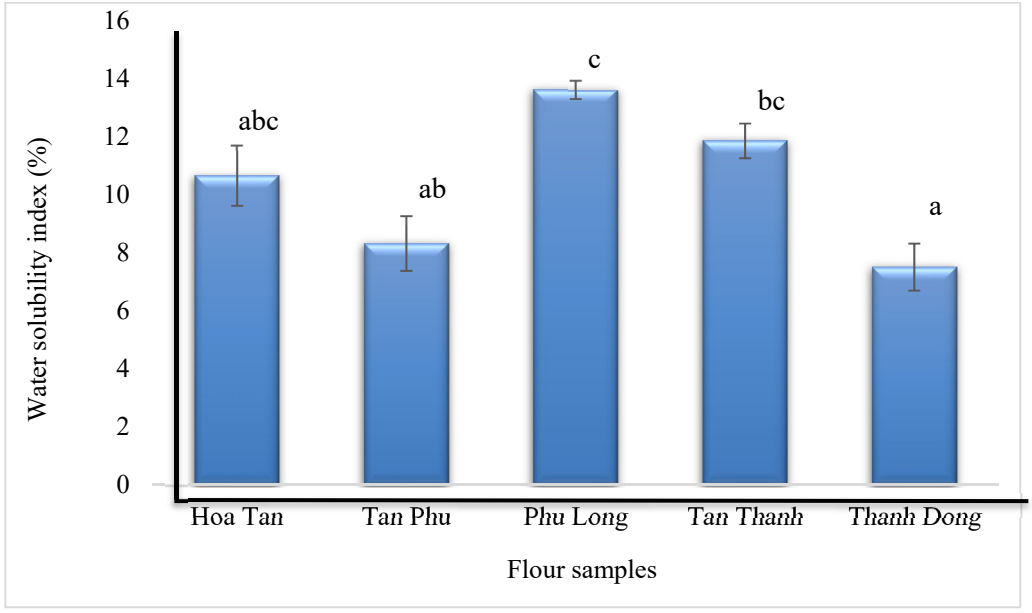

Fig. 2: Water solubility index (%) of purple sweet potato flours

Bars followed by the same superscript letter are not significantly different (P <0.05).

WSI of five purple sweet potato flour samples is presented in Figure 2. PL was found to have the highest percentage of WSI (13.6%) followed by TT (11.9%), while there no substantial discrepancy noted among HT, TP, and TD. The suitable explanation for WSI of these PSPF was mainly due to the difference in their amylose content and amylose-lipid complexation (Zuluaga *et al.*, 2007). Other research reported that higher hydrophilic constituents like polysaccharides and development in the amylose leaching and solubility of starch were also responsible for WSI (Chandra *et al.*, 2015).

Table 3: Pasting properties of purple sweet potato flours

Flour samples	Pasting temperature (°C)	Maximum viscosity (BU)	Final viscosity (BU)	Trough viscosity (BU)	Breakdown (BU)	Setback (BU)
HT	32.8 ± 1.4^{a}	1025 ± 9^{c}	940 ± 19^{a}	393 ± 31^{c}	632 ± 22^{b}	547 ± 50^{b}
TP	32.4 ± 0.8^{a}	740 ± 74^{b}	680 ± 75^{b}	360 ± 1^{bc}	381 ± 74^{a}	321 ± 76^{a}
PL	32.4 ± 0.8^{a}	676 ± 90^{b}	613 ± 6^{b}	265 ± 57^{b}	411 ± 33^{a}	349 ± 63^{ab}
TT	38.2 ± 0.1^{b}	453 ± 1^{a}	540 ± 53^{b}	116 ± 7^{a}	337 ± 8^{a}	424 ± 46^{ab}
TD	37.9 ± 0.6^{b}	458 ± 19^{a}	632 ± 42^{b}	140 ± 9^{a}	318 ± 10^{a}	493 ± 33^{ab}

HT: Hoa Tan; TP: Tan Phu; PL: Phu Long; TT: Tan Thanh; TD: Thanh Dong.

Data followed by the same superscript letter in the same column are not significantly different (P <0.05).

Table 3 provides the results of pasting properties of five purple sweet potato flour samples expressed as pasting temperature, maximum viscosity, final viscosity, trough viscosity, breakdown, and setback. The pasting profiles of these PSPF were considerably different. Among these flours, TT had the highest pasting temperature, while other pasting parameters including maximum viscosity, final viscosity, trough viscosity, breakdown, and setback of HT were the highest. Pasting temperature is vigorously associated to water absorption capacity of starch, in which maximum viscosity and breakdown reverberate the sensitivity of swollen granules to disperse approaching shear and final viscosity and setback demonstrate the inclination and manner of retrogradation of the starch gel (Afoakwa *et al.*, 2010). Thus, the paste of purple sweet potato from Hoa Tan village manifested the greater resistance against retrogradation, gel consistency and hot paste stability among other flours.

4 CONCLUSIONS

In this project, the bioactive compounds and physicochemical properties of purple sweet potatoes grown at different locations in Dong Thap and Vinh Long provinces were investigated. The results indicated that chemical compositions, bioactive compounds and physicochemical properties of these flours were significantly different. It is noticeable that purple sweet potatoes grown at Phu Long village (Chau Thanh district, Dong Thap province) had the highest amount of bioactive compounds and satisfied chemical compositions and physicochemical properties. Further study should be done to know the effects of soil composition and climate conditions on the quality of the purple sweet potatoes.

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Content and physicochemical properties of starches from different kinds of sweet potatoes grown in Dong Thap province

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ABSTRACT

Sweet potato (Ipomoea batatas L.) is an important agricultural plant grown in Dong Thap province to obtain tubers because of high starch yield. However, starch content and properties vary depending on genotype and growing conditions. The objective of this study was to determine content and physicochemical characteristics (chemical compositions, swelling power, viscosity and solubility) of starches obtained from two sweet potato samples (white and yellow sweet potatoes) from three locations in Chau Thanh district, Dong Thap province. On the dry matter basis, the starch content of sweet potatoes ranged from 49.8 to 66.8%, and the white sweet potato grown at Hoa Tan village had the highest starch content. On the wet matter basis, the starch content of sweet potatoes ranged from 16.1 to 20.4%, and the yellow sweet potato at Hoa Tan village had the highest starch content. The protein, fat, ash and total carbohydrate contents ranged from 0.15 to 0.25%, 0.07 to 0.14%, 0.15 to 0.22%, and 99.47 to 99.57%, respectively. The yellow sweet potato grown at Tan Phu village had highest starch swelling power at 90°C (15.42 g water/ g starch), while the yellow sweet potato from Hoa Tan village had highest solubility at 90°C (9.56%). In addition, starch suspension of the white sweet potato from Tan Phu village signified highest final viscosity and setback (626 and 390 BU, respectively), resulting in greatest resistance against retrogradation. The results of this study would provide useful information to select a high starch-content sweet potato practically grown in Dong Thap province for starch production.

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1 INTRODUCTION

Sweet potato (*Ipomoea batatas* L.), originated in Central America, but at present, is the seventh largest food crop generally cultivated all year round in various ecological habitats in many tropical and subtropical regions (Scott and Suarez, 1992). It is well known as a worldwide source of edible starch. Sweet potato supplies a considerable portion of the world's nourishment and is also an essential source for animal feed and industrial utilization.

Sweet potato which is well-known as a source of edible starch consists of around 6.9 to 30.7% of starch depending on the habitat in which they are grown (Liu *et al.*, 1985). Sweet potato starch is not only the most important ingredient in the human diet, but also a major industrial raw material for

paper, adhesives, pharmaceutical, plastics, textile, prepared food, and cosmetic industries (Mweta *et al.*, 2008). Sweet potato starch granules are round, oval or polygonal in shape and vary greatly in size of 2-42 μm. They are also designated as A or C type of crystalline structure, and their amylose content ranges in 18.7-20% (Hoover, 2001; Hung and Morita, 2005). Kaur *et al.* (2002) concluded that the environmental factors had some significant impacts on starch properties. However, the difference in physicochemical characteristics of sweet potato starches might affect the final quality of food products because starch is a major component and directly contributes to the functional properties and quality of food products.

The starch granules existing in the sweet potato tubers are implanted in cellulosic fibers and linked together by pectin substrates (Rahman and Rakshit, 2004). Thus, sweet potato starches in industrial scale are usually isolated by ultrasound pretreatment (Nandan *et al.*, 2014), mechanical disintegration of the cell wall and then utilization of water to wash starch granules out (Joshi and Kulkarni, 1993), or enzyme-assisted extraction method. Recently, there are more and more projects applied enzymatic treatments to enhance the recovery of starch from roots and tubers (Gayal and Hadge, 2003; Sit *et al.*, 2011).

According to Loebenstein (2016), Vietnam was the second largest producer of sweet potato in all over the world in 2015 with an estimated production of 1.45 million tons which is based on statistic data of Vietnam Ministry of Agricultural and Rural Development. Dong Thap province was the second largest production of sweet potatoes in Mekong Delta with the area of 39,300 ha (Ly Nguyen Binh *et al.*, 2014). In Dong Thap, white, yellow, and purple sweet potatoes are grown extensively at Hoa Tan, Tan Phu, and Phu Long villages of Chau Thanh District. However, little information of the starch yield of sweet potatoes grown in Dong Thap and their physicochemical properties, which are useful information for starch production and application have been reported. Therefore, in this research, the extraction yield and starch characteristics obtained from white and yellow sweet potatoes grown at three locations in Dong Thap province (Hoa Tan, Tan Phu, and Phu Long villages) were investigated.

2 MATERIALS AND METHODS

2.1 Materials

White and yellow sweet potato samples (*Ipomoea batatas* L.) used in this research were grown at Hoa Tan, Tan Phu, and Phu Long villages (Chau Thanh, Dong Thap, Vietnam). The two sweet potato samples were practically distinguished based on the color of skin and flesh. The vines were planted in July, 2016 and harvested in October, 2016. All the tubers in this experiment was in the uniformity of shape and size and did not contain any contamination including insects, smelly and rotten parts. After collecting, sweet potatoes were washed carefully and stored at 8 to 10^{o}C for further experiments. The white sweet potato sample from Tan Phu, Phu Long, and Hoa Tan villages were coded as W-TP, W-PL, and W-HT, respectively and the yellow sweet potato sample from Tan Phu, Phu Long, and Hoa Tan villages were coded as Y-TP, Y-PL, and Y-HT, respectively.

Commercial cellulase from *Aspergillus aculeatus* named Viscozyme Cassava C used in starch isolation was bought from a local agent in Ho Chi Minh City, Vietnam. Other chemicals were also purchased from a chemical store in District 10, Ho Chi Minh City, Vietnam.

2.2 Methods

2.2.1 Isolation of sweet potato starch

Starches were isolated from sweet potatoes by enzyme-assisted extraction, as a modified method of Benesi *et al.* (2004). These tubers after washing with water were peeled and sliced. Sliced sweet potatoes (100 g) was mixed with 150 mL of water. The mixture was then ground in a blender, and its pH was controlled around 5.5 – 6 before 3 mL of enzyme cellulase (100 U/mL) was added. After being incubated in a shaken water bath (125 rpm, 40^{o}C) for 3 hours, the mixture was added with 100 mL of water and filtered through a sieve with a cut-off size of 0.250 mm. After that, the solid residue was mixed with water, and the mixture was sieved again three times. Following this, all the filtrates were filtered with 0.105 mm-sieve, and then centrifuged at 3,500 rpm for 10 min. After all, the final supernatant was removed, and the solid residue was dried in the oven at 40^{o}C for 24 hours to reach 10-11% moisture content and pulverized into fine powder. Finally, the recovered capacity of starch was determined.

2.2.2 Determination of chemical compositions of sweet potato starches

Moisture content of sweet potato starches was determined using Moisture Balance Analyzer. The AACC approved methods 46-10, 30-10, and 08-01 (AACC, 2000) were used to analyze protein, lipid, and ash contents of sweet potato starches, respectively. Total carbohydrate content was calculated from the subtraction of protein, lipid and ash contents.

2.2.3 Determination of swelling power of sweet potato starches

Swelling power (SP) of sweet potato starches was measured based on the method of Sasaki and Matsuki (1998) with a minor modification. The starch suspension prepared from 0.16 g of starch samples and 5 mL of distilled water was placed in the falcon with coated screw caps. The mixture was heated at 50, 60, 70, 80, or 90°C and shaken continuously at 200 rpm for 30 min. After cooling to room temperature, the sample was centrifuged at 3,000 g for 15 min. The weight of sediment was recorded after carefully removing the supernatant. SP of starch (g water/ g starch) was calculated by dividing the weight of sediment by the initial weight of starch sample in dry basis.

2.2.4 Determination of solubility of sweet potato starches

The procedure written by Leach *et al.* (1959) was slightly modified and then applied to analyze the solubility of sweet potato starches. Starch sample (0.5 g) was suspended in 30 mL of distilled water. The mixture was heated at different temperatures from 50°C to 90°C at 10°C intervals for 30 min in a shaking water bath. After cooling to room temperature, the sample was centrifuged at 1,500 g for 30 min. Supernatant was dried at 120°C for 4 hours and then weighed. Solubility of starch (%) was calculated by dividing the weight of remained solid after drying supernatant by the initial weight of starch sample in dry basis.

2.2.5 Determination of pasting properties of sweet potato starches

Pasting properties of sweet potato starches were measured using a micro visco-amylo-graph (Brabender® GmbH & Co. KG, Germany). The starch suspension (8%, w/v) was preheated to 30°C, heated up to 93°C at a constant rate of 7.5°C/min and then held at 93°C for 15 min. Then, the paste was cooled to 30°C at the same rate and then held at 30°C for 15 min. The pasting properties of the slurry were recorded as the visco-amylo-graph program described as pasting temperature, maximum viscosity, trough viscosity, final viscosity, breakdown and setback.

2.2.6 Statistical analysis

All tests were performed at least in duplicate. Analysis of variance (ANOVA) was performed using the Tukey's test with significance level at $p < 0.05$ using SPSS software (SPSS Inc., USA). Correlation coefficients were also done using SPSS program (SPSS Inc., USA).

3 RESULTS AND DISCUSSIONS

3.1 Extraction yield of sweet potato starches

Table 1 illustrates the results of the dry matter content of six cultivars of sweet potatoes and their starch-extraction yield. The dry matter content of sweet potatoes ranged from 26.6 to 35.1% and the extraction yield of sweet potato starches was in a range of 16.1 to 20.4% in term of wet basis or 49.8 to 66.8% in term of dry basis. Among six cultivars of sweet potato, Y-HT accounted for the highest percentages of dry matter content (35.1%) and extraction yield of starch in term of wet basis (20.4%), while the lowest dry matter content (26.6%) and extraction yield of starch in term of wet basis (16.1%) belonged to Y-TP and W-TP, respectively. Furthermore, W-HT had highest percentage of extraction yield in term of dry basis (66.8%). Therefore, there was no correlation between dry matter content and extraction yield of starch. Dry matter of Turkish sweet potatoes was in a range of 29.2 to 51.1% depending on genotypes, growing location and environment (Yildirim *et al.*, 2011).

Table 1: Dry matter content and starch extraction yield of sweet potatoes

Samples	Color		Dry matter content (%)	Extraction yield (%)	
	Skin	Flesh		Wet basis	Dry basis
W-TP	White	White	31.1 ± 0.1^{c}	16.1 ± 0.8^{a}	51.6 ± 2.6^{a}
W-PL	White	White	35.0 ± 0.1^{e}	20.1 ± 1.0^{b}	57.4 ± 2.9^{b}
W-HT	White	White	28.9 ± 1.4^{b}	19.3 ± 0.9^{b}	66.8 ± 3.3^{d}
Y-TP	Purple	White-yellow	26.6 ± 0.9^{a}	19.8 ± 0.9^{b}	64.7 ± 3.2^{bc}
Y-PL	Purple	White-yellow	32.5 ± 0.5^{d}	16.2 ± 0.8^{a}	49.8 ± 2.5^{a}
Y-HT	Purple	White-yellow	35.1 ± 0.3^{e}	20.4 ± 1.0^{b}	58.0 ± 2.9^{b}

Data followed by the same superscript letter in the same column are not significantly different ($P < 0.05$) according to the Tukey's HSD test.

3.2 Chemical compositions of sweet potato starches

Proximate compositions of six cultivars of sweet potato starches are shown in Table 2. There was no remarkable difference in moisture content of sweet potato starches which was less than 11%. The chemical compositions of different sweet potato starch consisted of 0.15 – 0.25% of protein, 0.07 – 0.14% of lipid, and 0.15 – 0.22% of ash. Among six cultivars of sweet potato starches, W-TP had highest protein content (0.25%), W-HT accounted for greatest percentage of lipid content (0.14%), and Y-HT contained highest ash content (0.15%); while the lowest amounts of protein, lipid and ash belonged to W-PL, Y-HT, and Y-HT, respectively. However, there was no noteworthy discrepancy in total carbohydrate content of sweet potato starches which was in a range of 99.47 to 99.57%. Starch isolated from sweet potato without using enzyme consisted of 1.1% of protein, 0.9% of lipid, 0.1% of ash, and 97.9% of total carbohydrate (Hung *et al.*, 2014). However, in this research, pectin – cellulosic matrix of cell wall was broken down by enzyme cellulase, which resulted in the release of the starch granules and then gave higher yield without affecting the starch properties (Moorthy and Balagopalan, 1999). This leaded to the higher amount of total carbohydrate (99.47-99.57%) compared to other extraction methods without using enzyme.

Table 2: Chemical compositions of sweet potato starches (%, db)

Samples	Moisture content	Protein content	Lipid content	Ash content	Carbohydrate content
W-TP	10.73 ± 0.53	0.25 ± 0.01^{d}	0.08 ± 0.01^{a}	0.17 ± 0.01^{b}	99.50 ± 0.11
W-PL	10.81 ± 0.09	0.15 ± 0.01^{a}	0.12 ± 0.02^{b}	0.20 ± 0.03^{bc}	99.53 ± 0.14
W-HT	10.40 ± 0.39	0.17 ± 0.01^{a}	0.14 ± 0.01^{b}	0.22 ± 0.01^{c}	99.47 ± 0.17
Y-TP	10.42 ± 0.32	0.22 ± 0.01^{bc}	0.09 ± 0.02^{a}	0.22 ± 0.01^{c}	99.47 ± 0.11
Y-PL	10.58 ± 0.12	0.23 ± 0.01^{cd}	0.09 ± 0.02^{a}	0.18 ± 0.02^{b}	99.50 ± 0.12
Y-HT	10.50 ± 0.21	0.21 ± 0.01^{b}	0.07 ± 0.03^{a}	0.15 ± 0.01^{a}	99.57 ± 0.14
	ns				ns

Data followed by the same superscript letter in the same column are not significantly different (P < 0.05) according to the Tukey's HSD test. ns: non-significant.

3.3 Swelling power of sweet potato starches

Results for swelling power of six cultivars of sweet potato starches at different temperatures ranging from 50 to 90°C are shown in Figure 1. Swelling power of sweet potato starches considerably increased when heating temperature was between 70 and 80°C. Therefore, the data indicated that swelling power of sweet potato starches was not significantly different when heating temperature was lower than or equal to 70°C. Generally, swelling power was highest in Y-TP (15.42 g water/ g starch) at 90°C among six kinds of sweet potato starches, while the lowest SP belonged to Y-PL (11.52 g water/ g starch) at the same temperature. These outcomes were agreeable with the research by Gunaratne and Hoover (2002) showing that swelling power of starch had an uninterrupted escalation between the temperatures of 55 to 95°C. These differences in these swelling powers were mainly due to amylose content and its properties like amylose lipid complexation or total leached amylose in addition to phosphate content (Zuluaga *et al.*, 2007).

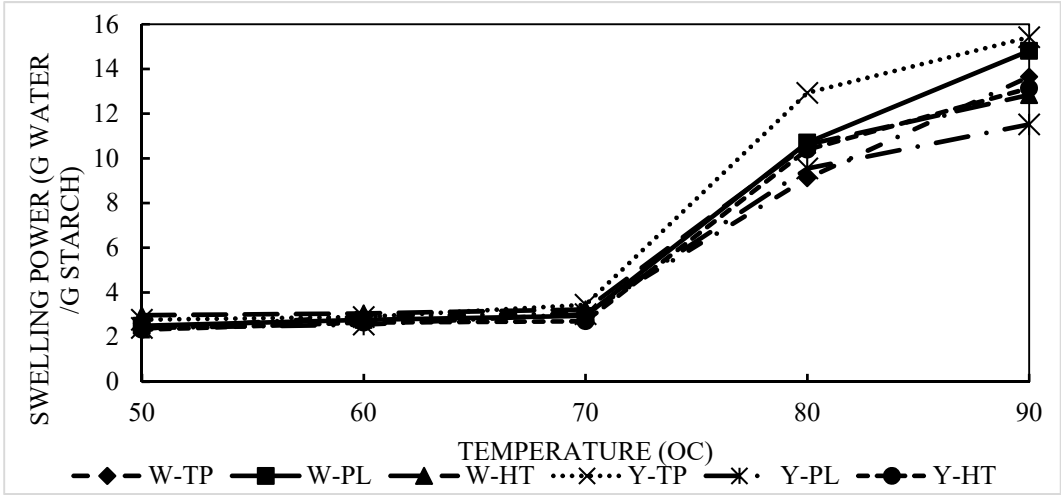

Fig. 1: Swelling power of sweet potato starches (g water/ g starch)

3.4 Solubility of sweet potato starches

Solubility of six cultivars of sweet potato starches are presented in Figure 2. The design witnessed in the solubilized attributes of six types of sweet potato starches was nearly the same as recognized from their swelling power. Their solubility considerably enhanced when heating temperature was higher than 70°C. Generally, the level of solubilization was highest in Y-HT (9.56%) at 90°C among six cultivars of sweet potato starches, followed by that of Y-TP, W-HT and then Y-PL, while the lowest amount of amylose leaching belonged to W-TP and W-PL (around 6.37%). These data corresponded with the research by Gunaratne and Hoover (2002) figuring out that solubility of starch elevated with a growth in temperature. These differences in solubility of sweet potato starches were mainly due to their amylose content and amylose-lipid complexation (Zuluaga *et al.*, 2007).

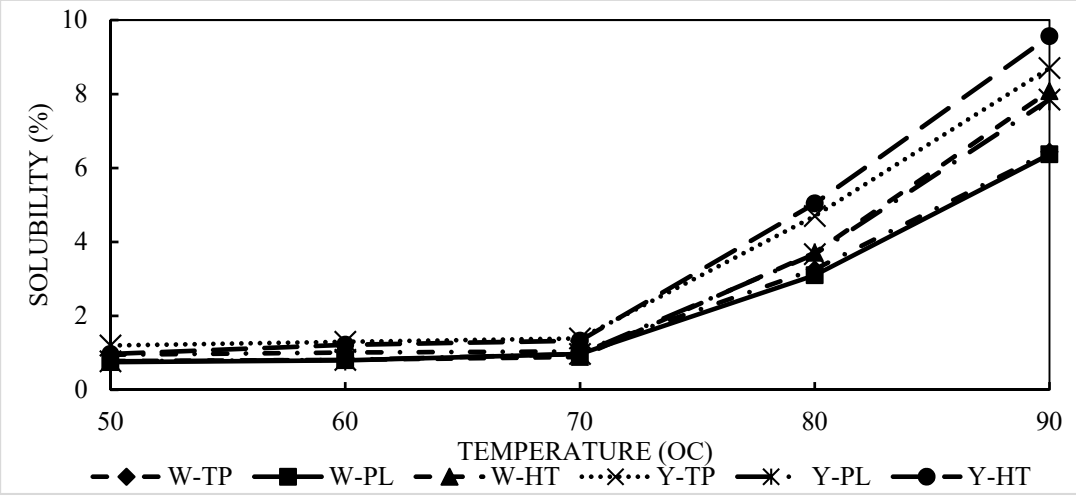

Fig. 2: Solubility of sweet potato starches (%)

3.5 Pasting properties of sweet potato starches

Pasting properties of six cultivars of sweet potato starches expressed as pasting temperature, maximum viscosity, final viscosity, trough viscosity, breakdown, and setback are demonstrated in Table 3. Generally, white sweet potato starches had higher pasting temperature as compared to yellow ones, and that of these starches ranged from 76.8 to 78.8°C. Among six cultivars of sweet potato starches, the maximum viscosity and breakdown of Y-HT (609 and 357 BU, respectively) were the highest, while W-TP had highest final viscosity and setback (626 and 390 BU, respectively). Maximum viscosity and breakdown reverberate the sensitivity of swollen granules to disperse approaching shear; and final viscosity and setback demonstrate the inclination and manner of retrogradation of the starch gel (Afoakwa *et al.*, 2010). Thus, among six cultivars of sweet potato starches, the paste of yellow sweet potato starch from Hoa Tan village manifested the highest gel consistency and hot paste stability, while the starch suspension of white sweet potato from Tan Phu village signified greatest resistance against retrogradation. The differences in these viscosity parameters were mainly due to their various amylose and protein contents (Hung *et al.*, 2007; Singh *et al.*, 2008).

Table 3: Pasting properties of sweet potato starch1,2

Samples	PT	MV	TV	FV	BD	SB
W-TP	78.3 ± 0.1^{c}	588 ± 4^{bc}	236 ± 1^{b}	626 ± 1^{d}	352 ± 4^{b}	390 ± 1^{e}
W-PL	78.6 ± 0.2^{cd}	582 ± 11^{b}	255 ± 5^{c}	569 ± 4^{a}	327 ± 7^{a}	314 ± 4^{a}
W-HT	78.8 ± 0.1^{d}	590 ± 4^{bc}	254 ± 2^{c}	607 ± 4^{c}	336 ± 5^{ab}	353 ± 3^{c}
Y-TP	76.8 ± 0.1^{a}	549 ± 9^{a}	220 ± 3^{a}	593 ± 7^{b}	329 ± 6^{a}	373 ± 4^{d}
Y-PL	77.2 ± 0.2^{b}	579 ± 11^{b}	225 ± 2^{a}	560 ± 6^{a}	354 ± 12^{b}	334 ± 7^{b}
Y-HT	77.3 ± 0.1^{b}	609 ± 7^{c}	218 ± 1^{a}	558 ± 4^{a}	391 ± 6^{c}	339 ± 5^{b}

[1]PT, pasting temperature (°C); MV, maximum viscosity (BU); FV, final viscosity (BU); TV, trough viscosity (BU); BD, breakdown (BU); SB, setback (BU).

[2]Data followed by the same superscript letter in the same column are not significantly different (P <0.05) according to the Tukey's HSD test.

4 CONCLUSIONS

In this project, white and yellow sweet potatoes grown at different locations in Dong Thap province had different dry matter content, extraction yield, chemical compositions, swelling power, solubility, and pasting properties of starch. The white sweet potato from Phu Long village and the yellow sweet potato starch from Hoa Tan village had the highest dry matter content and extraction yield. These sweet potatoes could be used for starch extraction with high efficiency. However, yellow sweet potato from Hoa Tan village should be examined more in the future.

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Quality changes and *in vitro* digestibility of bread substituted with tuber starches modified by citric acid and heat-moisture treatment

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ABSTRACT

Heat-moisture treatment combined with citric acid favors the escalation of resistant starch that is antagonistic to the small intestinal hydrolysis and plays an important role in reducing diabetes and giving positive effects on human health. The aim of this research was to examine the qualities (specific volume, textural properties, in vitro digestibility, and sensory profiles measured by descriptive analysis) of bread substituted with 20% of the mixture of citric acid and heat-moisture treated tuber starches (sweet potato, potato, and cassava) and vital gluten (9:1, w/w). An incorporation of 20% of modified starches and gluten into wheat flour for bread-making resulted in a substantial enhancement on resistant starch content and hardness and gumminess values, but a momentous fall on specific volume and sensorial profiles as well as overall acceptability of composite breads. Among three kinds of supplemented baking-products, breads complemented with 20% of modified cassava starch and gluten displayed intermediate resistant starch content (32.0%), and hardness value (14.94 N), but highest specific volume (3.34 cm^3/g), and score of overall acceptability (around 4.10/5.00) as compared to other modified starches.

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1 INTRODUCTION

Recently, the obesity rate has reached nearly one-third of the global population. Although there are various explanations for the development of obesity, the most crucial cause is especially diet (Chen *et al.*, 2010). Thus, the general recommendations for a healthy diet now are to reduce the intake of high cholesterol foods and to get more complex carbohydrates (Wolf *et al.*, 1999). However, the rate of starch digestion plays an important role in adjudicating the level of glycemic responses to dietary starches (O'Dea *et al.*, 1981). Therefore, a cost-effective dietary modification to lessen pervasiveness of obesity could be to follow a low-carb diet by improving the intake of ingredients containing high resistant starch content.

Due to diverse ecological habitats, roots and tubers, especially sweet potato, potato and cassava, are acknowledged as the most important food crops after grains. They can form new openings in food chain supply, and make a significant contribution to sustainable development and food security. However, they are mainly characterized as underdeveloped, small-scale with almost no postharvest techniques applied in Vietnam (Kim *et al.*, 2001). Thus, to prevent the post-harvest losses

and take advantage of being a source of edible starch, there are more and more projects conducted on producing starches from roots and tubers.

Based on the rate of digestion in the human small intestine, starches are divided into three groups: rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS) (Englyst *et al.*, 1992). RDS and SDS fractions are completely digested and absorbed in the human small intestine, whereas, small intestinal breakdown can be resisted by the total amount of starch that is defined as RS. RS plays an important role in reducing diabetes, lowering the risk of heart disease, and giving a positive effect on colonic health (Topping and Clifton, 2001; Sajilata *et al.*, 2006). Among four kinds of RS, RS type III occupies the most fraction and is easily produced by a hydrothermal method. Heat-moisture treatment combined with citric acid (CAHMT) can give a highest increase in the amount of SDS and RS content of the starches as compared to the native starch from sweet potato, yam, potato, or cassava and other treated starches (Hung *et al.*, 2014; Hung *et al.,* 2017).

The applications of RS in producing bakery are still extensively studying because the replacement of wheat in these products is a major technological challenge, and may give some remarkable effects on the specific volume, textural profiles and sensory qualities of the end-used products. In this research, 20% of composite flours of citric acid and heat-moisture treated sweet potato, potato, or cassava starch and vital gluten (9:1, w/w) was used to substitute for wheat flour in bread-making. In addition, effects of supplementation of composite flours for wheat flour on qualities (specific volume, textural properties, and sensory profiles) and *in vitro* digestibility capacity were also investigated.

2 MATERIALS AND METHODS

2.1 Materials

Potatoes (*Solanum tuberosum*) were grown in Da Lat city, Lam Dong province, and white sweet potatoes (*Ipomoea batatas* L.) were purchased at Hoa Tan village, Chau Thanh district, Dong Thap province. Cassava starch was produced and purchased from Hong Phat Cassava Processing Private Enterprise (Tay Ninh, Vietnam).

α-amylase from *Aspergillus niger* (28.75 U/mg) and amyloglucosidase from *Aspergillus oryzae* (300 U/mL) used in in vitro digestibility test were purchased from Sigma-Aldrich Company, while baking ingredients, VITEN wheat gluten, and other chemicals were purchased from local supplier in Ho Chi Minh City, Vietnam.

2.2 Methods

2.2.1 Starch isolation

Sweet potato or potato starch was isolated by repeated deposition method written by Lawal (2004). The starch released from the ground tubers was sieved through a series of sieves with aperture size of 0.25 mm and 0.125 mm. The final filtrate was settled down in 24 hours finally washed twice with tap water until the tailing fraction became negligible after settling. The isolated starch was dried in an oven at 40ºC for 24 hours (moisture content < 10%).

2.2.2 Hydrothermal treatment of starches

RS was produced by the combination of citric acid and heat-moisture treatment that was based on the study of Hung *et al.* (2014). Starch was mixed well with citric acid to achieve moisture level of 30% and heated at 110ºC for 8 hours. After heating, the mixture was neutralized with NaOH, settled and then centrifuged. Finally, the solid residue from centrifuging was dried at 40ºC for 24 hours and grinding was applied to achieve modified starch.

2.2.3 Bread-making method

The formula and procedures from method 10-10B (AACC, 2000) with a slight modification were applied to bake bread. The dough was prepared from 300 g wheat flour with or without 20% of composite flours of modified sweet potato, potato, or cassava starch and vital gluten (9:1, w/w), 18 g sugar, 4.5 g salt, 6 g dry baker's yeasts, and 187.8 mL water. After mixing in 15 min, the dough was fermented at 30ºC with humidity of 85% for 90 min, and punching was performed each 30 min. After 90 min of fermentation, the dough was divided into 3 pieces whose weight was around 130 g. Then, each piece was kneaded into a rounded shape for 15 min, and then it was laminated, rolled, cased off and placed in the pans and proofed at 38ºC with humidity was 90% for 33 min. Finally, the dough was baked at 180ºC for 20 min. After baking, the final product was formed. In order to determine *in vitro* digestibility, breadcrumb was dried at 50ºC for 24 hours and then pulverized.

Bread made from wheat flour was coded as WFB, while breads with 20% of mixture of modified cassava, potato or sweet potato starch and vital gluten (9:1, w/w) supplementation were coded as 20CSB, 20PSB, or 20SPSB, respectively.

2.2.4 Evaluation of specific volume and texture properties of starch-substituted bread

Specific loaf volume (cm^3g^{-1}) was determined by dividing loaf volume, which was measured by the

rapeseed displacement method (Giami *et al.*, 2004), by its corresponding loaf weight.

Textural properties of breadcrumb prepared in a rectangle shape (2 cm × 1 cm × 1 cm) were measured using a Zwitt/Roell Textural analyzer followed the method of Ulziijargal *et al.* (2013).

2.2.5 Evaluation of in vitro digestibility of starch-substituted bread

A minor modification in the method of Englyst *et al.* (1992) was used to measure RDS, SDS and RS of starch-substituted bread. The enzyme solution containing α–amylase (1400 U/mL) and amyloglucosidase (13 U/mL) was used to digest starch for determining the glucose content released in 20 min (G20) and in 120 min (G120). The remained solution after hydrolysis for 120 min was digested with amyloglucosidase (50 U/mL) to determine the total glucose content release (TG). G20, G120 and TG were used to calculate the content of RDS, SDS and RS.

2.2.6 Evaluation of sensory profiles of starch-substituted bread

Sensory qualities analysis was carried out based on the methods of Inglett *et al.* (2005) with moderate modification. The sensory tests were performed three times with an evaluation panel of 15 trained members. Testers were asked to score different kinds of breads in terms of crumb color, taste, aroma, appearance, texture, and overall acceptability by descriptive analysis (Table 1).

2.2.7 Statistical analysis

SPSS version 16 was used for one-way ANOVA of the results of qualities of breads. Tukey's test with significance level at $p < 0.05$ was used to compare the means of the results.

Table 1: Summary table of sensory evaluation of bread sample

Score	Color	Appearance	Texture	Odor and taste
5	Uniform color; typical golden brown crust of bread with creamish white crumb inside	Crust of bread has smooth surface with fully uniform porosity of crumb	Fully crispy crust of bread and spongy crumb	Fully pleasant aroma characteristic for bread, harmonious taste and mild sweet aftertaste
4	Relatively uniform color; golden brown crust of bread with creamish crumb inside	Crust of bread has smooth surface with slightly uniform porosity of crumb	Relatively crispy crust of bread and relatively spongy crumb	Pleasant aroma characteristic for bread, harmonious taste, mild sweet aftertaste
3	Non uniform color; golden brown crust of bread with deep cream white crumb inside	Crust of bread has slightly rough surface, slightly uniform porosity of crumb, but bigger air cells	Slightly crispy crust of bread and slightly spongy crumb	Aroma slightly characteristic for bread, very mild sweet aftertaste
2	Slightly dark brown crust of bread with slightly greyish crumb inside	Crust of bread has rough surface; non uniform porosity of crumb with many large air cells occurring	Hard crust of bread and soft crumb	Yeasty odor, salty or sour taste and no sweet aftertaste
1	Dark brown crust of bread with grey crumb inside	Crust of bread has rough surface, non-uniform porosity of crumb with too many large air cells occurring	Too hard curst of bread and too soft crumb	Off flavor, strange odor, no sweet aftertaste

3 RESULTS AND DISCUSSIONS

3.1 Cross-sectional view of starch-substituted breads

Figure 1 exhibits the cross-sectional view of breadcrumbs supplemented with 20% of mixture of modified tuber starches and vital gluten (9:1, w/w). The addition of modified tuber starches under CAHMT and vital gluten made the crumb structure smaller and more regular than WFB whose appearance had large and irregular gas cell. Generally, supplementation of modified starches containing high RS content did not have a remarkable depreciate impact on the external appearance and harmony of breads. Thus, these starches were suitable to supplement into wheat flour to make bread instead of fibers whose complementary addition could degenerate the color as well as appearance of breadcrumbs (Pomeranz *et al.*, 1977).

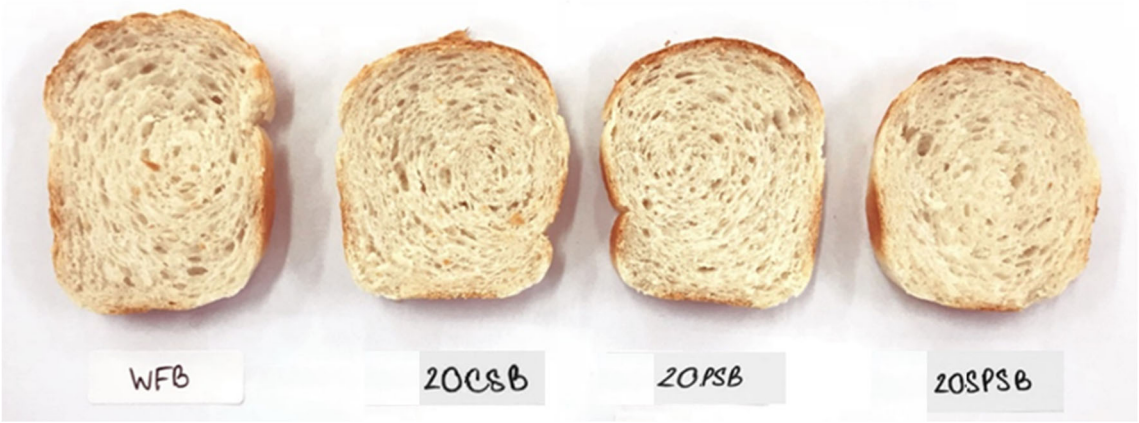

Fig. 1: Cross-sectional view of breadcrumbs supplemented with 20% of mixture of citric acid and heat-moisture treated cassava, potato or sweet potato starches and vital gluten (9:1, w/w).

WFB, bread made from wheat flour; 20CSB, bread with 20% of mixture of modified cassava starch and vital gluten (9:1, w/w) supplementation; 20PSB, bread with 20% of mixture of modified potato starch and vital gluten (9:1, w/w) supplementation; 20SPSB, bread with 20% of mixture of modified sweet potato starch and vital gluten (9:1, w/w) supplementation.

3.2 Specific volume and textural profiles of starch-substituted breads

Table 2 demonstrates data of the specific volume of four kinds of bread samples supplemented with modified tuber starches and vital gluten (9:1, w/w). Specific volumes of the starch-substituted breads ranged from 2.41 to 3.34 cm^3g^{-1}, which were lower than that of WFB (4.74 cm^3g^{-1}). Thereupon, these outcomes implied that a supplementation of tuber starches under CAHMT and vital gluten (9:1, w/w) for bread-making ensured a significant devaluation on the specific volume of breads. Among three kinds of starch-substituted breads, 20CSB had the highest specific volume (3.34 cm^3g^{-1}), while the lowest one belonged to 20PSB (2.41 cm^3g^{-1}). This result was agreeable to the research published by Miyazaki and Morita (2005) who revealed that a partial substitution of heat-moisture treated maize starch considerably reduced specific volume of starch-substituted breads. The aforementioned reduction of the specific volume of loaves was due to adverse effect of dilution on gluten content in the composite flour. The reduction of gluten content gave the significant effect on the dough properties including less elasticity and smaller extensibility resulting in smaller loaf volume (Makinde and Akinoso, 2014). Miyazaki and Morita (2005) also reported that the substitution of heat-moisture treated maize starch to wheat flour decreased the elasticity of the dough because the modified starch did not bind readily with gluten to form elastic dough, resulting in low specific volume of loaves.

The textural profiles of breadcrumbs with modified tuber starches and vital gluten (9:1, w/w) supplementation expressed as hardness, springiness, and gumminess values are exhibited in Table 2. Hardness and gumminess values of the starch-substituted breadcrumbs ranged from 12.07 to 15.96 N and 5.95 to 7.71 Nmm, respectively, which were higher than those of WFB (7.55 N and 4.10 Nmm, respectively). Thus, an incorporation of tuber starches under CAHMT and vital gluten (9:1, w/w) for bread-making resulted in a substantial increment in the hardness and gumminess values of breadcrumbs. Nonetheless, no noticeable inconsistency in the springiness value of all breadcrumbs was recognized. Thus, citric acid and heat-moisture treated tuber starch and vital gluten (9:1, w/w) substitution did not affect the rubbery characteristic of breadcrumb although these breads had a lower protein and gluten contents than WFB. These aforementioned data corresponded with the previous projects which revealed that a supplementation of chemically modified cassava or commercial resistant starch into wheat flour gave the remarkable increment in the hardness value of breadcrumb (Ozturk *et al.*, 2009; Rodriguez-Sandoval *et al.*, 2016). The considerable increase in hardness and gumminess values might be due to the higher amounts of solubilized amylose and short-chain molecules in these starches which easily retrograded after baking. In addition, Hung *et al.* (2005) also reported that the breads baked from flours which contained low protein content and gluten quantity were harder than breads baked from flours had higher protein content.

Table 2: Specific volume and textural profiles of breads supplemented with 20% of mixture of citric acid and heat-moisture treated cassava, potato or sweet potato starches and vital gluten (9:1, w/w)

Sample	Specific volume (cm^3g^{-1})	Textural profiles		
		Hardness (N)	Springiness(mm/mm)	Gumminess (Nmm)
WFB	4.74 ± 0.15^{d}	7.55 ± 0.46^{a}	0.91 ± 0.01^{a}	4.10 ± 0.34^{a}
20CSB	3.34 ± 0.19^{c}	14.94 ± 1.50^{b}	0.90 ± 0.01^{a}	6.92 ± 0.57^{bc}
20PSB	2.41 ± 0.15^{a}	12.07 ± 2.11^{b}	0.90 ± 0.02^{a}	5.95 ± 0.88^{b}
20SPSB	2.99 ± 0.04^{b}	15.96 ± 1.66^{b}	0.92 ± 0.01^{a}	7.71 ± 0.70^{c}

Data followed by the same superscript letter in the same column are not significantly different (P <0.05).

3.3 *In vitro* digestibility of starch-substituted breads

Table 3 illustrates the percentages of RDS, SDS, and RS in bread supplemented with modified tuber starches and vital gluten (9:1, w/w). Both RDS and SDS of starch-substituted bread ranged from 51.6 – 53.1% and 14.9 – 16.4%, respectively, which was lower than those of WFB (62.0% and 25.7%, respectively). Therefore, the percentages of RDS and SDS reduced remarkably when 20% of mixture of modified cassava, sweet potato or potato starch and vital gluten was substituted. The RS content of 20CSB, 20PSB, and 20SPSB were 32.0, 31.3, and 33.3%, respectively, which was higher than that of WFB (12.3%). Thus, RS content dramatically increased when there was an addition of modified cassava, sweet potato, and potato starch whose RS content in our earlier research was 40.9, 39.8, and 41.9%, respectively. These data corresponded to the work done by Ozturk *et al.* (2009) and Babu *et al.* (2015) where the addition of commercial resistant starch presented a massive increase in RS content of bread. In this study, these data also implied that RS in modified tuber starches not only persisted, but also formed more during baking period. Re-association of additional amylose and short-chain molecules of the starches which were gelatinized during baking and retrograded eventually were the major reasons for an enhancement in the amount of RS. This is because food processing, which involves heat and moisture like bread, in most cases, destroys RS type I and RS type II, but may form RS type III (Faraj *et al.*, 2004).

Table 3: Starch digestibility (RDS, SDS and RS fractions) of breads supplemented with 20% of mixture of citric acid and heat-moisture treated cassava, potato or sweet potato starches and vital gluten (9:1, w/w)[1,2]

Fractions	WFB	20CSB	20PSB	20SPSB
RDS	62.0 ± 2.0^{c}	51.6 ± 0.3^{a}	53.1 ± 0.2^{b}	51.8 ± 0.7^{a}
SDS	25.7 ± 2.7^{c}	16.4 ± 0.5^{b}	15.6 ± 0.8^{ab}	14.9 ± 0.8^{a}
RS	12.3 ± 0.8^{a}	32.0 ± 0.6^{b}	31.3 ± 0.6^{b}	33.3 ± 0.4^{c}

[1]*RDS, rapidly digestible starch; SDS, slowly digestible starch; RS, resistant starch.*

[2]*Data followed by the same superscript letter in the same row are not significantly different (P <0.05).*

3.4 Sensory evaluation of starch-substituted breads

Organoleptic properties of breads enriched with modified tuber starches and vital gluten (9:1, w/w) are demonstrated in Table 4. No conspicuous disagreement was marked between WFB and breads substituted with 20% of mixture of modified cassava or potato starch and vital gluten (9:1, w/w) in term of color, appearance, texture, odor and flavor, and overall acceptability. 20SPSB had significantly lower mean sensory score as compared to that of WFB in term of all sensory attributes, while no substantial discrepancy was also confirmed between 20CSB, 20PSB, and 20SPSB. This finding was very compatible with the works of Reed (2012) that observed bread with 20% RS from cooked rice replacement was best on overall acceptability judged by sensory panels. In addition, according to Majzoobj *et al.* (2014), supplementation of less than 30% corn resistant starch would give no significant influence on the sensory attributes of the cake, while a maximum level of corn resistant starch in cake recipe was concluded as an acceptable product amounted to 20%. Consequently, the result of the sensory evaluation pointed out that the substitution of up to 20% of mixture of citric acid and heat-moisture treated cassava starch and vital gluten gave moderately satisfactory overall acceptability (around 4.10 over 5.00).

Table 4: Mean sensory score of breads supplemented with 20% of citric acid and heat-moisture treated cassava, potato or sweet potato starches and vital gluten (9:1, w/w)

Sensory attribute	WFB	20CSB	20PSB	20SPSB
Color	4.37 ± 0.54^{t}	4.00 ± 0.74^{at}	4.03 ± 0.62^{at}	3.68 ± 0.72^{s}
Appearance	4.33 ± 0.78^{t}	4.13 ± 0.86^{at}	3.93 ± 0.74^{at}	3.71 ± 0.71^{s}
Texture	4.33 ± 0.62^{t}	4.03 ± 0.72^{at}	4.03 ± 0.70^{at}	3.68 ± 0.72^{s}
Odor and flavor	4.22 ± 0.80^{t}	4.20 ± 0.55^{at}	4.03 ± 0.72^{at}	3.75 ± 0.70^{s}
Overall acceptability	4.22 ± 0.80^{t}	4.10 ± 0.61^{t}	4.03 ± 0.62^{at}	3.64 ± 0.62^{s}

Data followed by the same superscript letter in the same row are not significantly different (P <0.05)

4 CONCLUSIONS

In this research, the supplementation of 20% of mixture of modified cassava, potato, or sweet potato starch and vital gluten (9:1, w/w) boosted the RS content in the bread as compared to that of WFB determined by *in vitro* digestibility method, but remarkably reduced the specific volume of bread loaves and increased the hardness and gumminess of breadcrumbs. Among three kinds of breads supplemented with sweet potato, potato, or cassava starch under CAHMT and vital gluten (9:1, w/w), 20CSB exhibited intermediate RS content, and hardness and gumminess values, but highest specific volume, and score of overall acceptability. Based on these aforementioned results, the substitution 20% of mixture of citric acid and heat-moisture treated cassava starch and vital gluten (9:1, w/w) into wheat flour had satisfied bread qualities, overall acceptability as well as health benefits.

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An investigation into English preparatory programs for EMI learning in higher education institutes in Vietnam

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ABSTRACT

In the era of globalization, the development of English as a medium of instruction (EMI) is of great interest to language and language policy researchers. Therefore, one of the main goals of the National Foreign Language Project launched by the Vietnamese Ministry of Education is to adopt EMI courses in all higher education institutes by 2020. However, in such a context that English is learnt as a foreign language in Vietnam, students' English proficiency is quite a great challenge for them to follow EMI programs. This strongly challenges students' learning and the effectiveness of EMI training. Therefore, this article critically examines the expectations of HEIs in Vietnam towards EMI students' English proficiency and how they prepare students with sufficient English for EMI learning. With a qualitative approach, this study employs document analysis method using purposive sampling to collect data in eight universities. The results show the inconsistency about the English entry each university requires and various practices of English preparatory programs for EMI learning. Based on the findings, recommendations are made to promote the effectiveness of the implementation of EMI programs in Vietnam and in similar contexts.

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1 INTRODUCTION

In the era of globalization, people need a common language to communicate with each other and access information, mainly in English. According to Naisbitt and Aburdene (1990), over 80% of all information stored in computers all over the world is in English, and more than half of technical and scientific journals and publications in the world are written in the English language. In addition, Vietnam's participation in the World Trade Organization in 2007 facilitated the global integration of Vietnam and created an ever-increasing demand for a workforce with multilingual competence, especially English language competence (Pham, 2011). To keep up with globalization, education systems and policies have been ceaselessly changed and improved to train labor resources with competitive capacities in international fields and the abilities to communicate in English.

Oh and Lee (2010) argue that English as a medium of instruction (EMI) or Teaching English through English (TETE) classes at universities have played

a positive role in lowering students' anxiety and enhancing students' English abilities by having students exposed to natural and authentic classroom. In fact, EMI in higher education is becoming more common all over the world (Mok, 2007 and Altbach *et al*., 2009) to boost university rankings (Rauhvargers, 2013), and a recognition that, in a globalized world, students need higher levels of English language competence if they are to compete on the international market (Graddol, 2006). In addition, factors such as the promotion of job mobility, employability, staff and student exchanges and joint- and double- degrees substantiate the case for English-taught degree courses (Fortanet, 2008).

Integrating with the international trend, in Vietnam, the Prime Ministerial Decision number 1400/QĐ-TTg (30 September 2008) is a national initiative on foreign language teaching and learning in the educational system from 2008 to 2020, known as the National Foreign Language Project 2020. According to Le (2012), by 2015 the project intends to begin EMI courses for 20% of university students in some disciplines and aims for all higher education institutes (HEIs) to adopt EMI courses by 2020. Indeed, 30 Advanced Programs were released by Vietnam's Ministry of Education and Training (MOET) at several higher education institutions nationwide from 2008 to 2015.

However, there have been worries and concerns about EMI programs. Local mass media report that English skills among undergraduates are a problem. A great number of graduates cannot communicate in English. In a notable study of to assess Vietnamese students' English skill, Vu and Nguyen (2004) researched on 1,000 students in five major universities in Ho Chi Minh City, the largest city of Vietnam. The results showed that Vietnamese students' language competency was insufficient to express their ideas or to communicate in everyday conversation, making them unable to understand materials from their lectures in English.

Another recent investigation from Da Nang University (2012) shows that 70% of the newly enrolled students have insufficient English proficiency to pursue studies in English. In addition, Vietnam is still ranked as a "low English proficiency" country (Education First, 2013). With such low English language competency, students would be unable to comprehend lectures or materials in English. This study is, therefore, of great interest to investigate how universities in Vietnam prepare students for EMI learning. Two research questions explored in the current study include:

1. What are the expectations of Vietnamese HEIs toward the English proficiency of EMI students?
2. What have the English preparatory programs provided for EMI learning in these institutes?

2 LITERATURE REVIEW

2.1 The English as Medium of Instruction Approach

The English as Medium of Instruction (EMI) is tracked back to the European content and language integrated learning (CLIL) approach, content-based teaching (CBT) and bilingual education in native English-speaking (NES) contexts. Macaro *et al.* (2018) define EMI as the use of the English language to teach academic subjects (other than English itself) in countries or jurisdictions where the first language of the majority of the population is not English. In this way, learners acquire both the subject content and target language in a natural setting (Sert, 2008). However, in Vietnamese pedagogy, this notion can be interpreted in different dimensions. For example, EMI may mean teaching all subjects in the curriculum in English comprising of subjects such as physical education, military education, Communism, Marxism, Ho Chi Minh thoughts. Yet, with EMI programs in Vietnam, only professional courses are taught in English and teachers of such listed courses above are free to use Vietnamese for instruction.

For an EMI program to be successful, Byun *et al*. (2011) identify three required factors comprising of students' and instructors' language proficiencies, the varying demands of different academic subjects and a facilitative body which can support EMI implementation. In the same vain, Kaplan and Baldauf (2005) propose that success "largely depends on policy decisions related to the teachers, the courses of study and materials and the resources to be made available" (p. 1014). However, these areas are developed differently in a particular nation "depending on how that nation's education system operates" (Kaplan and Baldauf, 1997, p. 217).

2.2 Challenges of EMI implementation

The fact that EMI requires instructions delivered in English leads to apparent obstacles. A review of literature reveals four major EMI challenges including teachers' language abilities, students' proficiency, methods, and inadequate resources. The first major challenge is lecturers' English abilities, particularly in contexts where English is a foreign language, and lecturers are non-native English speakers. This is in line with findings in

previous studies (Vinke *et al.,* 1998; Wilkinson, 2005; Kyeyune, 2010; Kennedy, 2011; Le, 2012 and Hamid *et al.*, 2013) showing that the inadequate English proficiency of EMI lecturers is one of the greatest hindrances for an EMI program to succeed. Vinke *et al.* (1998) report that content lecturers in the Netherlands had difficulty in expressing themselves effectively, especially in paraphrasing, searching for words, and refining statements, which results in detrimental effects on students' learning, such as less content coverage and knowledge loss. In the context of Israel, Shohamy (2012) claims that "It is often the case that academic professors will have high knowledge in one of the areas [content], not the other [language]" (p. 203). Indeed, to become a successful EMI instructor, there should be a combination of linguistic, academic and pedagogical competence, which few lecturers possess (Shohamy, 2012).

The second challenge for a success of EMI relates to the concerns of students' language abilities (Tsuneyoshi, 2005; Kyeyune, 2010; To, 2010; Byun *et al.,* 2011; Le, 2012). Kyeyune (2010) reports the frustrating communication failures in Ugandan classrooms because of students' low English proficiency. Another problem is a search for effective pedagogy. Wilkinson (2005) suggests that EMI can lead to effective content learning if instructional techniques (e.g. codeswitching between L1 and L2) are adapted, more time is allocated. However, unsupportive evidence for codeswitching is also found in other studies (Ibrahim, 2001; Shohamy, 2012; Mohamed, 2013). This controversy and limited literature on EMI pedagogy does not provide clear and appropriate teaching methods for effective EMI training.

The final challenge is limited resources (Le, 2012; Dang *et al.*, 2013). Baldauf *et al*. (2011) examine several Asian countries' language policies and conclude that "funding for normal programs, the training of teachers and money for textbooks are all inadequate" (p. 318).

In short, there have been multiple challenges in adopting EMI. It is undeniable that although each context may experience different problems. The next section examines the context of EMI in Vietnamese learning and teaching setting.

2.3 EMI in Vietnamese context

EMI is implemented in Vietnam through various forms of cooperation with international organizations and institutions, primarily in English speaking countries such as Australia, the UK and the US as well as some European countries where English is an established lingua franca. This cooperation model is founded on and reflects Vietnam's National higher education reform agenda, which supports collaboration with overseas institutions as key to the development and internationalization of Vietnamese HEIs (Government of Vietnam, 2005).

Overall, there are two main types of EMI programs namely advanced programs and high quality ones. However, the challenges inherent in the adoption of EMI at tertiary level in Vietnam discussed by Le (2012) and Vu and Burns (2014) is the lack of adequate linguistic competence of teachers and students has impeded the effectiveness of the EMI programs.

Bain (2004) identifies characteristics of a good college teacher, including good knowledge of his/her subject, possession of a long and impressive publication list, good ability to do scientific research, etc. However, the teaching staff in HEIs in Vietnam in general have both a shortage and weakness of professional knowledge. Specifically, the ex-president of Vietnam National University, in Hanoi, Nguyen (2004) claims that the teaching staff of HEIs are bookish, poor in professional knowledge and skills, lag behind the development of the modern world, are too old and suffer from inertia to keep up with the changing world. Due to language incompetence, a majority of academic staff are unable to read professional materials or journals in English to update their knowledge. Recent statistics show that on average, one Vietnamese professor only publishes 0.58 article in world-recognized refereed journals during a 10-year period (1996–2005) (VietnamnetBridge, 2008).

Regarding Vietnamese EMI students, Huong (2008, cited in Le, 2012) claims that due to their limited English skills, students do not dare to share ideas with other classmates or with lecturers. What they do is to sit in a place and listen to the lectures in a passive way. Since both teachers and students are not proficient in English, teachers are unable to deliver lectures in English. Likewise, students with poor language skills are unable to absorb the subject content. In addition to teachers' and students' insufficient English proficiency, a number of potential difficulties have arisen when implementing the EMI program at HEIs in Vietnam, which needs more scientific research in this field.

2.4 Related studies on the importance of students' English proficiency for effective EMI learning

Kang and Park (2005) state that students' appropriate level of English proficiency is a requirement for the successful implementation of an

EMI policy. This was strongly demonstrated through the strong positive correlations between students' English fluency and their understanding of textbooks and lectures and between their English fluency and performance in EMI classes. Kang and Park (2004) also suggest that the school should provide a variety of EMI support programs such as undergraduate English writing classes and preparation classes for EMI in major areas.

In Turkey, Kirkgöz (2009) investigated teacher and student perceptions about the effectiveness of English language instruction in an EMI university in Turkey. The results showed that over 90% of students were not adequately prepared English to learn academic subjects through EMI. In addition, Akyel and Özek (2010) used questionnaires and interviews with both EMI teachers and students in a single Turkish university and found that the teachers focused more on reading and listening as the important skills to be developed. However, both teachers and students felt a neglect on speaking skills which allowed students to operate successfully in their undergraduate EMI programs.

Kim (2014) also highlighted the problem of students' insufficient English ability for EMI. Over 40% of undergraduate students and 28% of graduate students were ill-equipped for EMI classes. Despite students' insufficient English abilities, appropriate measures have not been taken to develop students' English proficiency. Kim (2014) also suggested that in order to help enhance students' English language skills, the instructors may provide feedback on students' English problems in their oral or written work with the help of English professors.

Thus, for successful EMI, the HEIs must focus on developing and improving their students' English skills first and foremost. Indeed, Baker & Jones (1998) raise the importance of a bridging program with an argument that the more demanding the curriculum area, the higher the level of learning expected, and the later switch to learning through a second language, the more important it is to provide bridging programs. In this sense, the English preparatory programs are considered to be an important bridging program, supporting students with sufficient English abilities for EMI learning. However, very few studies are conducted to examine how universities prepare their students English proficiency for effective EMI learning so far, especially in the context of Vietnam, making this present study more timely and significant.

3 METHOD

This study employed qualitative approach using document analysis of university regulations for the EMI programs as a method to collect data. A purposive sampling approach (Riffe *et al.*, 2005) was used to locate sources for content analysis. These sources must meet certain criteria: (1) the university websites; (2) EMI implementation or programs called as advanced or high quality programs; (3) higher education contexts, and (4) descriptions of the English preparatory programs for EMI learning. Having outlined these criteria, sources were located by exploring databases on universities websites.

Through the purposive sampling approach, a total of eight universities' websites were identified to match the criteria comprising of Hanoi Foreign Trade University, Center for advanced educational programs-Hanoi National Economics University, Vietnam Maritime University, Ho Chi Minh National University – University of Information Technology, Ho Chi Minh City University of Technology, The University of Da Nang - University of Science and Technology, VNU University of Economics and Business, Banking University of Ho Chi Minh City and Can Tho University. Data were analyzed in terms of four emerging themes comprising of the name of the university, the names of the advanced and high quality programs, English proficiency requirements for entrance to these programs and English proficiency requirement for graduation.

4 RESULTS

The data show that there are two main types of EMI programs comprising of high quality and advanced programs, in which the former has more diversity in majors than the later. Even some universities have the high-quality programs only, namely Banking University Ho Chi Minh City, VNU University of Economics and Business, Ho Chi Minh City University of Technology and The University of Da Nang - University of Science and Technology. In addition, the majors or disciplines of these EMI programs also differ from one university to another due to its specializations. Among the investigated universities, Ho Chi Minh City University of Technology, the University of Da Nang - University of Science and Technology and Center for advanced educational programs – Hanoi National Economics University have the most various majors with more than 10 disciplines whereas the other universities just have two or three EMI majors. The tables below show more information on the English requirements for EMI students as well as the English preparatory programs to meet the learning outcomes in these eight major universities that have offered EMI programs in Vietnam.

4.1 Information on the English requirement eligible for entering EMI learning

Information about the compulsory English entry for EMI students is illustrated in Table 1.

In terms of the entrance English requirement, only three universities comprising of Ha Noi Foreign Trade University, Banking University of Ho Chi Minh City and Can Tho University show specific English proficiency in terms of international standards which students have to obtain to be allowed to register for EMI programs. Especially, Hanoi Foreign Trade University has a detailed requirement of the English proficiency level prior to entering the EMI programs, which calls for B2 V-step (Vietnamese Standardized Test of English Proficiency), TOEIC 500, TOFEL iBT 60 or IELTS 5.0 as a compulsory condition for students to enter the EMI programs accompanied with student interviews in English about general knowledge, expectations and goals for learning in the advanced and high quality programs. This is also the highest English requirement as opposed to other universities, followed by Banking University of Ho Chi Minh City. The least demanding is Can Tho University, at just A2-CEFR, IELTS 3.0 or TOEIC 400. Meanwhile, Center for advanced educational programs – Hanoi National Economics University and VNU University of Economics and Business just have a kind of placement test to measure students' English abilities, which students must reach a certain score to be allowed for EMI learning. The other three universities either require students' specific English competence or reveal no information in this area.

Table 1: Information on the English requirement eligible for entering EMI learning

No.	Institutes	Entrance English requirements
1.	***Hanoi Foreign Trade University***	***B2 V-step, TOEIC 600, TOFEL (paper-based) 450 IELTS 4.5 or pass the University's English entrance exam.***
2.	Center for advanced educational programs – Hanoi National Economics University	English Test, Essay Writing and Interview
3.	VNU University of Economics and Business	English Test (4/10) and x2
4.	Ho Chi Minh National University – University of Information Technology	No information
5.	Ho Chi Minh City University of Technology	Not required
6.	***Banking University of Ho Chi Minh City***	***TOEIC 450***
7.	The University of Da Nang - University of Science and Technology	No information
8.	***Can Tho University***	***A2 CEFR, IELTS 3.0, TOIEC 400, TOEFL ITP 337, TOEFL iBT 31, KET 70, PET 45.*** Or English placement Test: 36 marks at least

4.2 Information on the English preparatory programs for EMI learning

The English preparatory programs the investigated universities prepare for EMI students' learning are shown in Table 2.

Generally, the table indicates the inconsistency in the ways each university prepares their students with English competence eligible for EMI learning. Specifically, Ho Chi Minh City University of Technology and Banking University of Ho Chi Minh City designed a curriculum for the English preparatory program in terms of IELTS format which comprises of 4 levels from foundation to upper-intermediate. Depending on the students' English background, they are placed in an appropriate course or level. Similarly, Banking University HCMC built five English preparatory courses accounting for 20 credits which students learn in five consecutive semesters to gain V-step (B2) or IETLS 5.5. Meanwhile, The University of Da Nang - University of Science and Technology employs TOEFL iBT. They even cooperate with AMA Foreign Language Center to train students in EMI programs to obtain TOEFL iBT targeted at the score of 61.

Apart from Hanoi Foreign Trade University and Can Tho University, the remaining universities do not reveal any information on the type of test format employed to design the curriculum for the English preparatory programs. Another interesting thing is that there are some differences in the number of credits and courses for English learning among universities. Among them, Center for advanced educational programs – Hanoi National Economics University have the most credits (36), with figures for other universities ranging from 15 to 20 credits in their English preparatory programs. Another noticeable point is that students will be equipped with more English learning hours in advance programs as

compared to high quality ones since advance programs often have associated education with foreign prestigious universities, requiring students with higher English proficiency.

Table 2: Information on the English preparatory programs for EMI learning

No.	Institutes	English preparatory programs
1.	Hanoi Foreign Trade University	***16*** credits, 6 English courses
2.	Center for advanced educational programs – Hanoi National Economics University	High quality programs: 18 credits Advance programs: **36** credits
3.	VNU University of Economics and Business	***19*** credits, 4 courses
4.	Ho Chi Minh National University – University of Information Technology	2 courses
5.	Ho Chi Minh City University of Technology	4 courses (IELTS format) from elementary to upper-intermediate levels
6.	Banking University of Ho Chi Minh City	***20*** credits, 5 courses (IELTS format)
7.	The University of Da Nang - University of Science and Technology	Cooperate with AMA Foreign Language Center (TOEFL)
8.	Can Tho University	***20*** credits, 9 courses

The English preparatory programs can be found in two universities. In the first case, the high quality program disciplined in Business Administration at Hanoi Foreign Trade University shows six English courses in which the first four courses have three credits each and involve in General English and in the rest two ones, each has two credits and focus more on Business situations. These English courses are taught by lecturers at the English for Specific Purposes Department. Detail descriptions of these courses are shown below.

The English 1 and 2 courses aim to provide students with the basic concepts and terms of business English together with the focus on listening and speaking practice to prepare students to perform effectively in English language tests in the form of IELTS, TOEFL or other 6-level language ability tests in Vietnam. The textbooks include *Pathways Reading, Writing and Critical Thinking 2, The Business 2.0* Pre-Intermediate (3rd Edition Student Book) and *Effective Academic Writing 2* for English 1 course. The English 2 course utilize four main books comprising of *Pathways Listening, Speaking, and Critical Thinking 2, The Business 2.0* (B1+ Intermediate) as main materials and *Skillful Listening & Speaking 2, English Pronunciation in Use* (Intermediate) and *Business Vocabulary in Use* (Elementary to Pre-intermediate) as compulsory reading books.

The English 3 course, in contrast, has more focus on advanced reading and writing skills so that students are able to use English effectively in different situations not only at work but also in daily life and meet the requirements of the standard equivalent output B2-CEFR. This course also expands and improves business English vocabulary for students. The textbooks include *Pathways Reading, Writing and Critical Thinking and The Business 2.0* 2rd Edition B2 Upper-Intermediate. The English 4 course is the last course of the general English program designed for high quality students at advanced level, aiming to increase students' abilities in writing and speaking Business English through business skill-related speaking tasks and written business communications skills. The textbooks are the in-house material named as *English for Business – Speaking and Writing*, *In company 3.0 Advanced* together with articles in the field.

The English 5 course is designed not only to provide students with useful specific knowledge in Business English but also to equip them with the written competence in common letters and business correspondence such as: inquiries and replies, offers and quotation, orders, complaints, adjustments, application letter and an effective professional CV for their job application. Textbooks encompass *Oxford Handbook of Commercial Correspondence*. Oxford University Press and compulsory readings such as *Oxford Handbook of Commercial Correspondence* and *The Language of Business Correspondence in English*. The English 5 course equips students with specialized knowledge in the Contractual English language from the processes of negotiation, drafting and interpreting the contract content. Two books used in this course include *Exporting and the Export Contract* and *Contract Law for Paralegals.*

Thus, the English preparatory programs for high quality students at Hanoi Foreign Trade University cover both General English that still covers technical English vocabulary in the field and general Business - oriented situations. Meanwhile, the English preparatory program at Can Tho University

mainly targets at improving students' General English only. To be more specific, there are nine courses accounting for 20 credits comprising of 2 Listening & Speaking courses, 2 Writing courses, 2 reading courses, a Grammar course, a Pronunciation course and an English presentation course. The English skills courses employ Skillful 2 course book together with some in-house material preparing students for V-step exams. The other courses use materials designed by staff members at School of Foreign Languages, Can Tho University.

4.3 Information on the English requirement for students' graduation in EMI programs

The English requirements or the expected English proficiency EMI students have to achieve at the end of the EMI programs are illustrated in Table 3.

Table 3: Information on the English requirement for graduation in EMI programs

No.	Institutes	English requirement for graduation
1.	***Hanoi Foreign Trade University***	***TOEFL iBT 100 or TOEFL 600 paper-based)***
1.	Center for advanced educational programs – Hanoi National Economics University	TOEFL PBT 500; V-step (B2); IELTS: 5.0
3.	VNU University of Economics and Business	V-step 4/6 (B2); IELTS 5.5; TOEFL 500; or TOEIC 600
4.	Ho Chi Minh National University – University of Information Technology	High quality programs: TOEFL PBT 450 Advance programs: TOEFL PBT 550 or IELTS 6.0
5.	Ho Chi Minh City University of Technology	IELTS >= 6.0; TOEFL iBT ≥ 79
6.	Banking University of Ho Chi Minh City	V-step 4/6 (B2) or IETLS 5.5
7.	The University of Da Nang - University of Science and Technology	TOEFL iBT 61
8.	Can Tho University	B2 V-step

It can be seen from the table that there was the inconsistency among universities about the English requirements for EMI students to be graduated. Ha Noi Foreign Trade University has the most demanding English proficiency, at TOEFL iBT 100, which is equivalent to IELTS 7.0, followed by Ho Chi Minh City University of Technology, at nearly a band lower i.e. IELTS 6.0. Other universities require a slight mutual difference in students' English proficiency such as the levels of B2 (V-step), IELTS 5.0, IELTS 5.5, TOEFL 500, TOEIC 600 and so on. In addition, the English requirement for graduation in the advanced program is higher than that in the high-quality ones. This is TOEFL PBT 550 or IELTS 6.0 as opposed to TOEFL PBT 450 as shown in the table for Ho Chi Minh National University – University of Information Technology. Especially, in the case of Can Tho University, EMI students are only expected to obtain B2 V-step when they finish learning four academic years. In other words, the strict or compulsory regulation on EMI students' English proficiency prior to their graduation may vary from one university to another.

5 DISCUSSION

There are two main types of EMI programs comprising of high quality and advanced programs in higher education institutes in Vietnam. However, the current study shows the inconsistency in the ways each university prepares English for their EMI students as well as their expectation about their students' English ability eligible for graduation. To be more specific, some universities have specific requirements about the students' English ability prior to EMI learning whereas the others mainly let students do a placement test to classify students and put them in appropriate pre-university courses. In this respect, Ha Noi Foreign Trade University has the highest requirement, followed by universities in Ho Chi Minh city, whereas Can Tho University situated in the Mekong Delta requires students with the least English proficiency to be eligible for EMI learning. This certainly reflects the discrepancy about the English proficiency among different regions in Vietnam as well as the teaching quality.

Another noticeable point is that each university has its own curriculum for the English preparatory programs in terms of the number of credits for English courses, types of test format to follow and coursebooks. Although not all universities show the detailed curriculum and syllabus for each English course in the English preparatory programs, there are two different orientations can be clearly seen. One curriculum just focuses on building up students' English general knowledge according to a certain type of test format chosen. The other one covers both general English and technical English terms or vocabulary together with basic knowledge or situations in specific disciplines so that students

can be well prepared for studying EMI subjects later in the program. Therefore, students learnt with the former curriculum may encounter certain difficulties when they move to major subjects.

Similarly, the English requirement towards EMI students' graduation also differs from one university to another. This reflects the differences in the education quality as well as prestige of each university, leading to certain discrepancies in students' job or higher education opportunities. Although there are some inconsistencies in the ways the examined universities prepare English ability for EMI students, it shows their great consideration and effort to enhance Vietnamese students eligible for EMI learning. This idea is quite congruent with Byun *et al.* (2011) suggesting that there should be an establishment of an English threshold and improve students' English proficiency; otherwise, students suffer from both language and content loss in EMI environments (Kyeyune, 2010; Mohamed, 2013).

In addition, though the MOET has a general regulation for EMI students to obtain B2- VSTEP English proficiency or equivalent, with the discrepancies among universities in ways of recruiting students and the English requirement eligible for EMI learning, this strongly affects the curriculum designs and the quality of the English preparatory programs, leading to the effectiveness of EMI programs generally. Therefore, the MOET should legislate another common agreement on a certain general English standard required for EMI students prior to their entrance to EMI learning as well. Hamid and colleagues argue that if EMI is to be institutionalized, attention needs to be paid to the language-in-education policy areas of students and evaluation (Hamid *et al.*, 2013).

Moreover, in such a limited English speaking environment as in Vietnam, creating opportunities for EMI students to practice English with native speakers and outside the classroom should be taken into great consideration. Therefore, native speakers should be involved in teaching English preparatory courses for EMI students so that students can build up confidence in communicating and learning with foreign lecturers in subsequent subjects in their disciplines and in real life. Addition to taking English courses specifically oriented to academic teaching in a formal setting, EMI students should be offered opportunities to get engaged in more informal activities, such as study tours in English-speaking countries, scholar exchanges, and travel grants for international conferences (Wilkinson, 2005; Ball and Lindsay, 2013).

6 CONCLUSIONS

Two main types of EMI programs comprising of high quality and advanced programs are found in the eight examined universities in Vietnam. The findings also indicate the inconsistency in the ways each university prepares their students with English abilities eligible for EMI learning. Specifically, some universities employ IELTS or TOEFL, and some do not provide any information or have specific test format when designing the English preparatory courses. The discrepancies are also figured out in the number of credits spent to foster EMI students' English ability learning ranging from 18 to 20 for high quality programs and 20 to 36 for advanced programs. It also means that there are more English investments in advanced programs. The universities' expectations towards EMI students' English ability for graduation also differ from one university to another. In fact, EMI has a vast influence on aspects of life as it determines who will participate in power and wealth (Tsui and Tollefson, 2004). With such discrepancies and inconsistencies found, the Vietnamese MOET should take greater consideration into planning and implementing language policy to reinforce EMI programs throughout the nation.

This study is, however, still limited in the way that it just qualitatively analyzes data through available websites. The findings may therefore not cover details about the English preparatory programs in all of the examined universities. Future studies should be conducted with more universities and research tools such as interviews with EMI administrative staff to gain more insight into the issue. In addition, studies on the effectiveness of the English preparatory programs on students' EMI learning are also worth investigating in order to find ways to maximize the training quality and reinforce the sustainable development of EMI programs in similar contexts.

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Evaluation of the oxidation stability of jatropha biodiesel/diesel blends

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ABSTRACT

One of the major technical issues facing biodiesel is its susceptibility to oxidation which is due to its content of unsaturated fatty acid chains, especially those with bis–allylic methylene moieties. In addition, the presence of air and other factors also influences the oxidation process of biodiesel including presence of light, elevated temperature, as well as extraneous materials such as metals which may be even present in the container material. The overall objective of this work is to evaluate the oxidation stability of Jatropha biodiesel/diesel blends. An acid–catalyzed pretreatment followed by a standard transesterification procedure in a potassium methoxide solution to produce Jatropha methyl ester (JME) from Jatropha curcas L. oil (JO) with high acid value of 16.25 mg KOH/g was accomplished. The analysis of the physicochemical properties showed JME demonstrated potential as a good candidate for feedstock in biodiesel production because the studied physicochemical properties of JME adequately satisfied the relevant standards for biodiesel quality, with the exception of the kinematic viscosity at 40°C. Also, gas chromatography–mass spectrometry analytical result showed that fatty acid composition of JO was quite similar to that of conventional oils. Especially, the evaluation of oxidation stability of Jatropha biodiesel/diesel blends was accomplished with respect to the change in the quality after oxidation by bubbling oxygen at elevated temperature as well as oxidation of blend fuels in contact with copper plate. The results demonstrated a strong correlation between biodiesel concentration and blend stability; i.e., the increase in biodiesel concentration results in the lower stability in both cases of the copper strip corrosion test and the accelerated oxidation.

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1 INTRODUCTION

Global climate change and fossil fuels depletion are among the most critical issues facing human civilization. The development and use of renewable energy sources have the potential to address both issues. Among these sources, biofuels have been gaining increasing interest in the past decade as a substitute for petroleum in the transportation sector to mitigate the effects of greenhouse carbon dioxide (CO_2) emissions on climate change and offset the depletion of fossil fuels. However, the challenges with biofuels are that their production must be highly scalable and result in minimal environmental

impact. Moreover, their physicochemical properties must be consistent with those of petroleum. Known to be renewable, biodegradable and less pollutant emission, biodiesel, which is prepared from vegetable oils and animal fats, has been studied extensively as an alternative for diesel fuel.

Although biodiesel can be used in its neat form, it is recommended that being used in its blends with petrodiesel at any level leads to the improvement in its quality. Its blend is denoted as "BXX", where "XX" represents the biodiesel fraction (i.e., B20 consists of 20% of biodiesel and 80% of petrodiesel). However, the differences in chemical nature between biodiesel and diesel may lead to differences in the physicochemical properties, affecting engine performance and pollutant emissions. Therefore, the quality control of biodiesel blends should be monitored in several aspects (Benjumea *et al.*, 2008; Jha *et al*., 2008). Oxidation stability of biodiesel has been the subject of extensive studies, primarily in regard to oxidation during extended storage periods. This is one of the major challenges for the use of biodiesel because of its unsaturated fatty acid content, which is especially susceptible to oxidation (Knothe, 2006). There are numerous factors, such as presence of air, elevated temperatures and presence of metals that were found to promote oxidation. According to Knothe and Dunn (2003), the increase in the number of double bonds in biodiesel enhances its oxidation.

The presence of metal, such as copper, can increase the oxidation of biodiesel. Sarin *et al*. (2009) studied the influence of metal contaminants on oxidation stability of JME and reported that even low concentrations of metal contaminants resulted in nearly the same influence as high one. Of all studied metals, copper was found to be the strongest detrimental and catalytic effect on oxidation stability of biodiesel. The oxidative stability significantly decides the fuel quality; therefore, the European Committee for Standardization (CEN) was mandated to develop standard specifications and test methods regarding the biodiesel oxidation stability. This issue has been addressed in the European FAME (free fatty acid methyl ester) standard EN 14214 and in the recent version of the European standard for automotive diesel (EN 590:2009). The latter requires the determination of oxidation stability of Jatropha biodiesel/diesel blends using the modified Rancimat method EN 15751. The finished blends of diesel fuel with biodiesel shall comply with a minimum induction period of 20 hrs. at 110ºC. The established European standard EN 14112 using a Rancimat apparatus is the test method for determining oxidative stability of biodiesel at 110ºC, with a minimum induction period of 6 hrs. However, in order to ensure a high quality biodiesel within the EU, the CEN has already discussed a change in the limit of a minimum induction period of 8 hrs instead of 6 hrs.

Jatropha curcas is a drought–resistant shrub or tree grown in Central and South America, Southeast Asia, India, and Africa. *Jatropha curcas* was propagated from South America to other countries in Africa and Asia by the Portuguese (Gubitz *et al*., 1999). It is well adapted to arid and semiarid regions and often used for soil erosion control. The seeds of Jatropha resemble castor seeds, somewhat smaller in size (0.5 to 0.7 g) and dark brown in color. The oil content of the seed varies from 30 to 40%. The oil is toxic due to the presence of diterpenes, mainly phorbol esters, responsible for tumor–promoting activity.

Oil contents, physicochemical properties, fatty acid composition and energy values of Jatropha species were investigated in many studies (Banerji *et al*., 1985; Kandpal and Madan, 1995; Haas and Mittelbach, 2000; Kumar *et al*., 2003; Pramanik, 2003; Akintayo, 2004; Shah *et al*., 2004) while it is considered that oil from Jatropha has toxic substance (Hirota *et al*., 1988; Gandhi *et al*., 1995; Makkar *et al*., 1998; Abdel Gadir *et al*., 2003). A number of research papers have appeared on the production of biodiesel from Jatropha curcas L. oil (JO) (Berchmans and Hirata, 2008; Lu *et al.*, 2009; Shutt *et al*., 2010). So far, however, there has been little work about the oxidation stability of blending biodiesel from nonedible oilseeds like Jatropha with diesel.

Biodiesel fuels are expected to be used in various forms of blends, thus the quality standardization is necessary. The objective of this research to investigate the oxidation stability of Jatropha biodiesel/diesel blends (B5, B10, and B20) prepared from Jatropha methyl ester (JME). The effects of evaluated temperature (115ºC) and copper metal on the oxidation stability of Jatropha biodiesel/diesel blends were studied further in this work.

2 MATERIALS AND METHODS

2.1 Materials

All the chemicals used (for the biodiesel fuel production or analyses) were analytical reagent grade or equivalent.

JO was obtained from West Nusa Tenggara, Indonesia.

2.2 Methods

2.2.1 Conversion of oil into biodiesel

The used crude oils in this study contained a high concentration of free fatty acids. Therefore, methyl esterification of the free acid was carried out in methanol in the presence of sulfuric acid (1 w/v%) prior to transesterification for biodiesel production, according to method of Ghagde and Raheman (2005). The pre–processed crude oil was then mixed with potassium methoxide solution (1.0 w/v) in a round glass flask at the volume ratio of 1:8 (alkaline methanol to oil), and the mixture was kept to react at 60°C while stirring. After 2 hrs., the product was cooled down and left for separation of the biodiesel in a separating funnel. After removing the glycerol layer at the bottom, the remaining biodiesel was washed twice with an equivalent amount of water to remove residual methanol and alkali. Finally, the biodiesel was vacuum–dried at 60°C until reaching the water content of less than 500 ppm, which is the standard limit of water content.

2.2.2 Fatty acid profile

Fatty acid methyl ester composition was analyzed by a gas chromatograph–mass spectrometer (GC–MS 2010, Shimadzu Co., Japan) equipped with a wax column (Inert–Cap Pure Wax column, 30 m × 0.25 mm–id, GL Sciences Inc., Japan). Helium was used as the carrier at a flow rate of 2 mL/min. One µL of FAME samples was injected through the auto injector (AOL–20i, Shimadzu), and the peaks were observed under the column temperature programmed to start at 50°C, being heated to 260°C at a rate of 5°C/min and held at this temperature for 10 min. The compounds in a biodiesel fuel were identified by the mass spectroscopic peak profile and the molecular weight of parent peak using the chemical library in the workstation system equipped in the present Shimadzu GC–MS apparatus.

2.2.3 Physicochemical properties

Density of the biodiesels and the feedstock oil was determined using a density meter (DMA 4100A, Anton Paar GmbH–Austria).

The kinematic viscosity (40°C) test was carried out using a glass capillary viscometer and evaluated according to American standard ASTM D6751 and JIS K 2390–2008. The kinematic viscosity specifications (determinations at 40°C) in biodiesel standards which are 1.9–6.0 mm^2/s in the American standard ASTM D6751 and 3.5–5.0 mm^2/s in the JIS K 2390–2008. The kinematic viscosity specifications in petrodiesel standards are 1.9–4.1 mm^2/s (No. 2 diesel fuel, to which biodiesel is usually compared; No. 1 diesel fuel is 1.3–2.4 mm^2/s) in the American standard ASTM D975 and minimum value of 1.7 mm^2/s in the JIS K 2204. The viscosity of biodiesel is slightly greater than that of petrodiesel, which is reflected in the specifications in the standards.

Water content was determined by Karl Fisher coulometric titration method using 831 KF coulometer (Metrohm) according to BS EN ISO 12937:2001.

Free fatty acids are formed by the oxidation as well as the hydrolysis of biodiesels, and the degree is usually given in the acid value. Acid value is the parameter showing the content of free acid, and is expressed as the amount of KOH in milligram to neutralize the free acid (mg KOH/g of sample). Acid value was determined by the titration method using potentiometric titration apparatus (Model AT–610, Kyoto Electronic Manufacturing Co Ltd., Japan).

Iodine value is a measure of the total number of double bonds in fat or oil (or its derivatives). Iodine value is given in the amount of iodine which reacts with the unsaturated bonds in oil or fat, and the unit is given in gram of I_2/100 g of sample. Iodine value is analyzed according to the standard of JIS K0070–1992. Briefly, biodiesel is reacted in Wijs reagent and thereafter with an aqueous KI solution. The liberated iodine is then titrated with a 0.01mol/L standard solution of sodium thiosulphate.

Oxidation stability in terms of induction period, expressed in hour unit, was determined following the EN 14112 method using of Rancimat test apparatus (Model 743, Metrohm). Oxidation stability was also identified by the oxygen adsorption method of PetroOXY, operated at 120°C (Petrotest GmbH & Co. KG, Germany), and the oxidation stability data were expressed in the unit of hour and minutes (h:m).

Copper is susceptible to corrosion, and it is used as an indicator of the corrosiveness of a fuel. The copper strip corrosion has also changed physicochemical properties of fuel. Corrosion test was carried out and judged by the surface appearance after the immersion of copper plates in fuels. In the standard of JIS K 2513, a copper plate was immersed in fuels at the temperature of 60°C for 3 hrs, and the appearance of copper at the surface is used for judgment. In this study, the polished copper strip is immersed in a specific volume of biodiesel sample and B5, B10, B20 blends, and kept under at 60°C for 3 hrs, 24 hrs, 120 hrs and 164 hrs, and at 115°C for 3 hrs, 164 hrs to observe the change in the quality. At the end of the treatment, the copper strip was removed, and physicochemical properties of

biodiesel samples were analyzed in terms of oxidation stability, peroxide value, kinematic viscosity at 40°C, and iodine value. Accelerated oxidation method was also employed to evaluate the oxidation stability of Jatropha biodiesel/diesel blends. Oxidation of these fuels was carried out using TOS–10T apparatus (Yoshida Scientific Ltd., Japan) according to JIS K 2514. A 350 mL of filtered middle distillate blended fuels are aged at 115°C for 3 hrs and 16 hrs under bubbling oxygen at a rate of 3 L/h.

3 RESULT AND DISCUSSION

3.1 Characteristics of JO

The physicochemical properties and fatty acid compositions of the JO feedstock are summarized in Table 1.

Table 1: Characteristics of the JO

Test	Unit	Value
Density at 15°C	g/cm^3	0.9200
Kinematic viscosity	mm^2/s	34.99
Water content	mg/kg	980
Acid value	mg KOH/g of oil	16.25
Iodine value	g I_2/100 g of oil	98.0
Fatty acid composition	%	
Palmitic acid (C16:0)		13.37
Stearic acid (C18:0)		5.45
Oleic acid (C18:1)		45.79
Linoleic acid (C18:2)		32.27
Others		3.12
Average molecular weight		847

Composition and proportion of fatty acids in the oil depend on the plant species and the growth conditions. The oil used in this study contains 18.82% of saturated acids (palmitic acid and stearic acid) and 78.06% of unsaturated acids (oleic acid and linoleic acid). The difference in unsaturated content of different raw material would strongly affect the properties of biodiesel produced, especially its oxidation stability. JME, containing high degree of unsaturated carbon, tends to have low oxidation stability. Average molecular weight of JO was calculated based on the fatty acid methyl ester composition of the JME which was identified by GC–MS. The acid value of JO was found to be 16.25 mg KOH/g of oil (free fatty acid corresponds to 8.13%).

3.2 Analysis of key characteristics of JME and its blends

JME was selected because JO is one of the most popular feedstock for biodiesel production at present. Analysis of the physicochemical properties of JME indicated that the produced biodiesels demonstrated potential as candidates to be considered for feedstock in biodiesel production because they exhibited fuel properties within the limits prescribed by the latest American Standards for Testing Material, European standards and Japanese Industrial Standard. However, JME is unsuitable in pure state for its direct use as fuel in internal combustion engines because its kinematic viscosity at 40°C (6.6 mm^2/s) is higher than that of the upper limit of international standard (6.0 mm^2/s). Thus, JME was blended with reference diesel. Biodiesel can be used as a blend with diesel in any proportion; however, according to ASTM D975 and D7467, the maximum proportion of biodiesel is 20% (ASTM D2274, 2005). Biodiesel and diesel are not chemically similar, accordingly, biodiesel is composed of long–chain fatty acid methyl esters, whereas diesel is a mixture of aliphatic and aromatic hydrocarbsons containing approximately 10 to 15 carbons. Because of their differences in chemical compositions, it is not surprised that biodiesel and diesel exhibit different fuel properties. The blend also exhibits some its own properties, which are different from neat biodiesel and diesel fuels. Specifically, the most important fuel properties influenced by blending of biodiesel with diesel are oxidative stability. Therefore, the focus of this work is to evaluate oxidation characteristics of JME blends and compare JME blend properties with those of neat JME. This method is also used for studying the effects of blending ratio to the characteristics of biodiesel diesel mixture.

It is predicted that the oxidation stability of JME will increase by blending with diesel, as hydrocarbon constituents of diesel are more stable to oxidation than FAME (especially in the case of unsaturated FAME). As shown in Table 2, the increase in JME proportion led to significant increases in peroxide value, acid value, and kinematic viscosity at 40°C while decreases in induction period (IP). These results demonstrated that blending with diesel really improved oxidation stability of biodiesel.

Table 2: Analytical data of JME and its blends

Specification	Diesel	B5	B10	B20	B100	Standards for B100		
						ASTM	EN	JIS
Rancimat, h	–	33.5	23.55	16.27	5.68	6.0	3.0	10.0
PetroOXY, h	–	6.17	5.25	4.43	1.34	–	–	–
Acid value, mg KOH/g of sample	0.14	0.0005	0.0019	0.017	0.141	0.5 max	0.5 max	0.5 max
Peroxide value, meq/kg	–	1.0	1.3	2.1	11.7	–	–	–
Iodine value (g I_2/100 g of sample)	–	9.59	14.90	–	97.39	120 max	120 max	120 max
Kinematic viscosity at 40°C (mm^2/s)	3.07	2.95	3.1	3.31	6.6	1.9–5.0	3.5–5.0	3.5–5.0

3.3 Evaluation of the oxidation stability

The stability of the blends is mainly affected by the characteristics of the diesel fuel. Higher refining base diesel with lower sulfur content decreases oxidation stability of the final blend.

The absence of sulfur in the base diesel, which acts as a natural oxidation inhibitor, and the presence of antioxidant additive in the methyl ester have strong effect on the stability of the final blends. Diesel fuel containing catalytically–cracked compounds may be more unstable, compared to hydrotreated diesel fuel. The oxidation stability of these blends was investigated from the two aspects, namely the oxidation under bubbling oxygen at 115°C, and the contact with copper metal plate.

3.4 Oxidation in contact to copper

The appearance of copper surface was practically almost unchanged; however, the fuel quality changed significantly, depending on the immersed conditions.

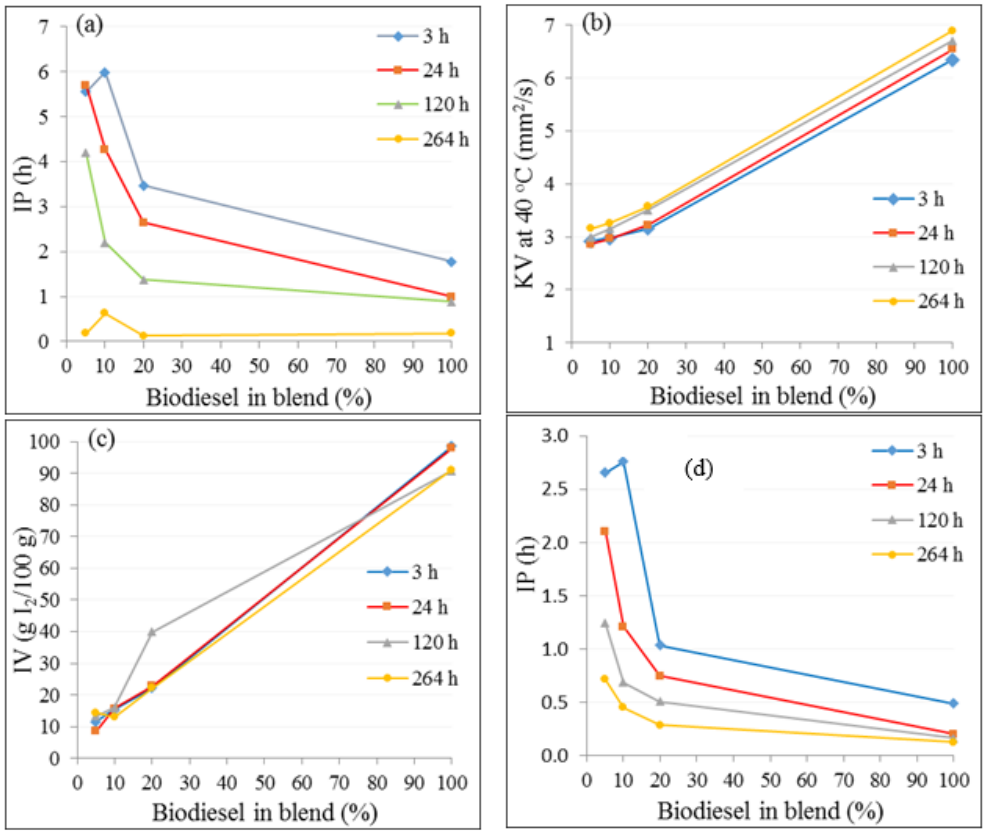

Fig. 1: Results of copper strip corrosion test at 60°C with different time treatment: Rancimat test (a), kinematic viscosity at 40°C (b), iodine value (c), and PetroOXY (d)

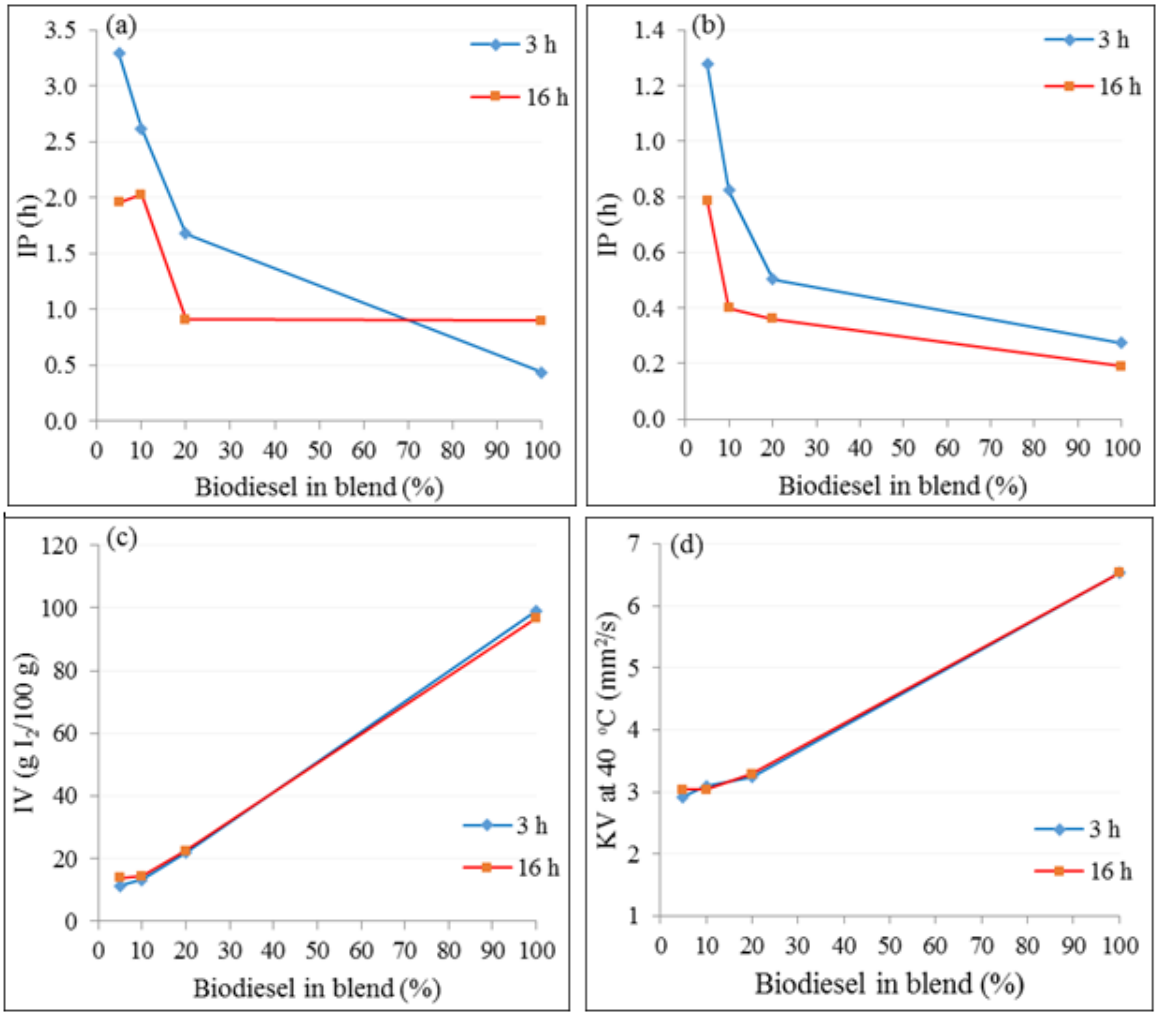

Fig. 2: Results of oxidation stability after strip corrosion test at 115°C: Rancimat test (a), PetroOXY test (b), Iodine value (c), and kinematic viscosity at 40°C (d)

The results revealed the fuel quality changed corresponding to blending ratio, with the higher proportion of JME would negatively affect the oxidation stability (Fig. 1). Moreover, the oxidation of the blends remarkably increased under the contact with copper surface. It is also found that the degree of oxidation correlated with the increase in temperature and contact time (Fig. 2). The increase of peroxide value and the decrease of IP clearly demonstrated for this correlation.

3.5 Accelerated oxidation

Accelerated oxidation method was employed to evaluate the oxidation stability of Jatropha biodiesel/diesel blends. It is suggested that the change in acid value before and after the oxidation can be used as standard indicator for the oxidation. In this study, however, peroxide value was measured before and after the accelerated oxidation, respectively. In addition, the oxidation stability was also examined by monitoring the change in IP. As shown in Fig. 3, peroxide and acid values increased dramatically, indicating the decrease in oxidation stability after the accelerated oxidation. The quality of the Jatropha biodiesel/diesel blend is, in theory, naturally dependent on the blending ratio. The result obtained from the experiments (Fig. 3) is consistent with the theory. Remarkable changes in quality was found in blends with biodiesel proportion ranging from 5 to 20% after the oxidation.

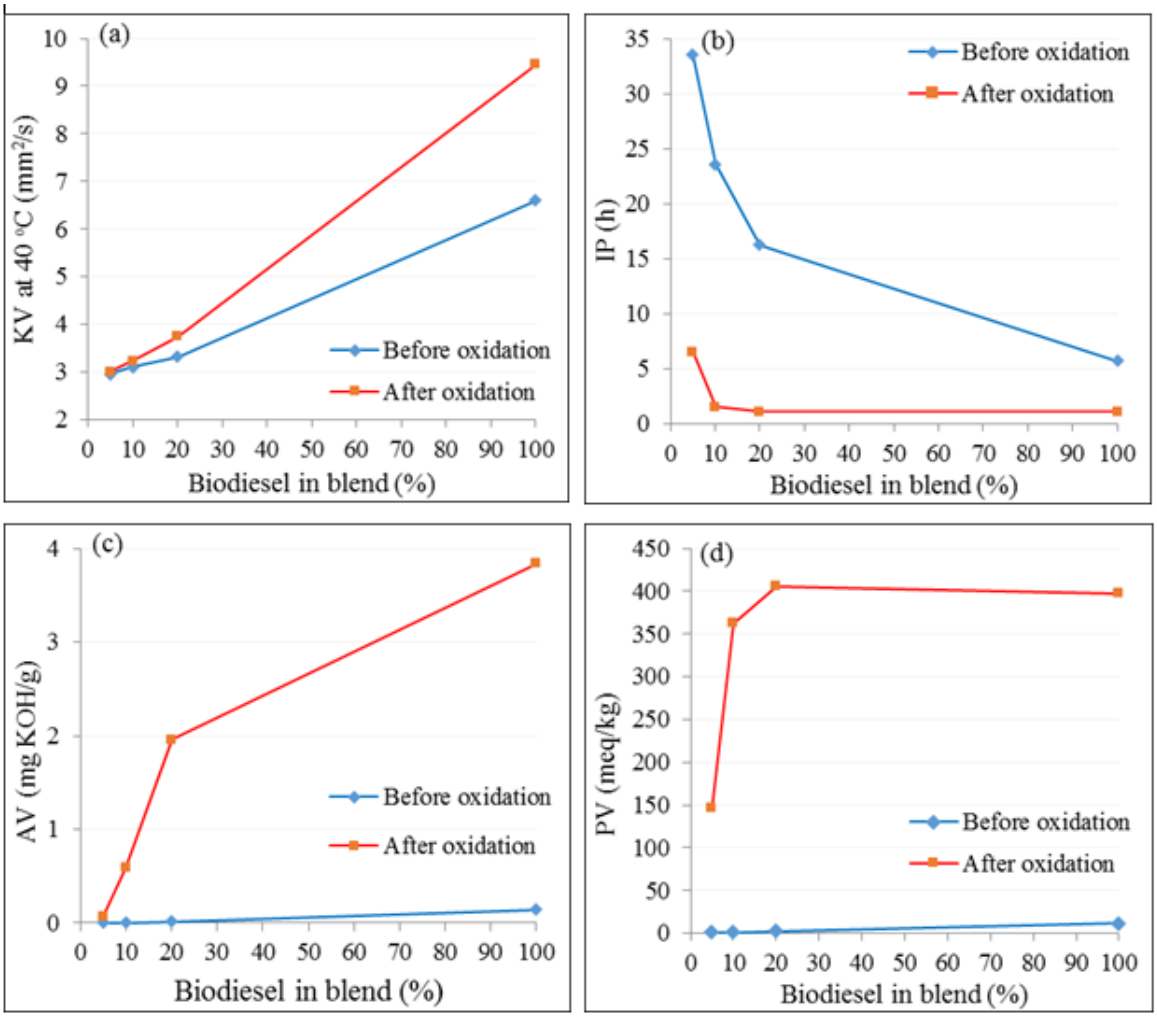

Fig. 3: Results of kinematic viscosity at 40°C (a), Rancimat test (b), acid value (c), and peroxide test (d) before and after the oxidation test at 115°C for 16 hrs. under bubbling oxygen

4 CONCLUSIONS

In summary, the stability of biodiesel is affected by various parameters. In this study, a correlation relationship between the blending ratio and the stability of Jatropha biodiesel/diesel blend was observed. This relationship is demonstrated by the change of IP, iodine value, acid value, peroxide value of Jatropha biodiesel/diesel blends, compared with both neat biodiesel and diesel. In general, the increase in Jatropha biodiesel/diesel ratio results in the lower stability of the blend in both cases of the copper strip corrosion test and the accelerated oxidation. The observation from this study provided useful information for the penetration of biodiesel into the transportation sector.

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Effect of the surface mounting technology assembly based on lean production: A case study

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ABSTRACT

Lean production is a popular technology which is applied widely and brings a lot of benefits. Many electronics companies in Vietnam apply this technique to eliminate wastes and enhance effectiveness. This paper is to present a study on the application of lean production on a Surface Mounting Technology (SMT) line. In this research, the SMT assembly line with a traditional batch production model would be transferred to lean production-oriented model. This work was begun by realizing the current state value stream mapping and then identifying wastes to establish an implemented plan. Reengineering of the SMT line focused on the aspects of reduction such as reducing wastes, standardizing works, and improving the layout. Some positive results were recorded such as increasing about 50% productivity per each shift, decreasing lead time from 6 days to 192.76 seconds; moreover, controlling the end-line quantities in a day was fixed. Furthermore, the application results are discussed especially.

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1 INTRODUCTION

Over the past several decades, many industrial zones have been established, and factory companies have been newly invested, leading to a trend of industrial development. The global modernization has been creating competition among enterprises more and more fiercely. There are a lot of problems in the field of technology, of which two issues that affect competitiveness are production cost and product quality. There are always issues at workplaces such as a large number of semi-finished products at workstations and production bottlenecks caused by the deficiency of synchronization. To improve the competitiveness of enterprises themselves in the context of global competition as severe today, they must constantly improve their production line. Lean manufacturing is not a new technology but has been studied and applied in many countries such as Japan, the Republic of Korea, USA, and many European countries. Lean was introduced by an engineer after the occurrence of World War II, and firstly designed for production lines of Toyota Company, or Toyota Production System (Ohno, 1982). Philosophy of this system is to eliminate wastes, empower human resources, reduce inventory, and importantly meet customer demands. Instead of storing required resources for future production, Toyota Company has built up a good relationship with suppliers. In addition, by training multi-skill workers, the company could arrange them in flexible ways; therefore, it could meet the unstable customers' demands better than competitors could. Lean methodologies are a compilation of many

techniques, which was used by many companies in the past. The difference is the consolidation of these techniques into one set of powerful methodologies and their applications. Specifically, they are a series of techniques that allow producing one unit at a time, and at a formulated rate while eliminating none of adding-value waiting and queuing time or other delays. Lean technology is a systematic approach method to maximize customer requirements at the highest level as well as minimize wastes.

In Vietnam, numerous studies have consistently found that value stream mapping (VSM) leads to improvement in aspect of lean production system. The implementing VSM process are to find out the main causes of waste such as the difference between the output, the daily target, work in process (WIP) at many stages and the unreasonable layout. In the future, researchers will come up with several methods to eliminate wastes and improve efficiency and effectiveness. A recent study clearly showed various benefits of VSM, providing opportunities for Clipsal Vietnam Co with improving productivity (Phong *et al*., 2015). East West Industries (EWI), which belongs to East West Manufacturing group, established construction for its first Vietnam facility in April 2008 with the main types of production, being called an assembly, injection molding, metal stamping, pad printing, warehousing, and offices. This paper is to improve the competitiveness of the company. With a strong focus on quality, engineering, and satisfying our customers, EWI is poised to continue to deliver competitive manufacturing or domestic and international customers. To put it another way, this study will provide the steps of lean implementation in detail, and proposes the STM processing improvement line to reduce the wastes.

2 LITERATURE REVIEW

Lean technology is a systematic approach method to maximize meeting the demand of customer at the highest level as well as minimize wastes. VSM is one of the tools that is a key tool to identify the cause of waste in the process and steps can be taken to reduce or eliminate it. This philosophy was first introduced by the researchers who argued that eliminating waste was the biggest goal the system wanted to enhance (Womack *et al*., 1990). VSM is developed to overview the value processing of lean manufacturing systems. Other report introduced an overview of the VSM; the steps involved in making it and analyzed the causes of waste in all processing (Wade and Hulland, 2004). A case study of the "Lean" approach was presented, using the main tool of the value stream to draw a simulation model of the production line at a steel company (Abdulmalek and Rajgopal, 2007). This study gives the potential benefits of this tool, reducing producing time and inventory time. Especially, a case study in India had applied a VSM to enhance the streamlined operations to minimize cycle time in the production process (Seth *et al*., 2008). The report showed that VSM has proven effectiveness in identifying and eliminating wastes according to the basis producing processes, namely assembly facilities. VSM is used as an advanced tool to improve the supplier's productivity in the automation industry. The researchers presented the current data collection and the current VSM that analysing the actual wastes, proposed the specific changes to the lean production model (Yu *et al*., 2009). In addition, a systematic approach based on the technical value of the VSM was developed to identify the current processes. Besides, layout design is a very important issue in production, management, and control. Balanced peanut included the assignment of tasks required to handle a product, to the allocation of machinery so that idle time is minimized (Dolgui and Gafarov, 2017). Balanced line should be done in most production lines, but bottleneck nodes often occur. There were many simple balancing methods that bring efficiency and quality of the production line to be shown in productivity and balance indexes (Lam *et al*., 2016). In addition, cellular layout is performed after the problem has been solved. In the cellular layout system, the machines have grouped into multiple cells, each dedicated to a specific family, and the goal is to maximize the independence of cellular manufacturing system (Pattanaik and Sharma, 2009). Kleiner (2006) argued that engaging in larger system components such as organizational design and management was not a novel for chemists; however, it was built by providing the specific methods and tools result in large-scale results. To improve production efficiency, other authors looked at the causes of job stress reduction as stress and management; moreover, technical solutions could be used to improve human performance (Murray and Thimgan, 2016). Special considerations have given to minimize the risks associated with human fatigue, error checking and reliability analysis. In the context of increased computerization and automation, which have caused changes in human work in production, the report showed that human factors and interactions between humans with the machine affect the design of production systems (Becker and Stern, 2016). In this article, the research provided a list of human tasks in the present and future cyber-physical production system (CPPS), including a trend estimate for the decline, increase, or change of

these additional tasks. Finally, the authors combined the findings with expert assessments of CPPS trends and recent employment data from the German job market. In addition, a report has also reviewed the theories and motor control factors that affect the performance of motion and indicated that internal movement changes are part of the complete mission (Gaudez *et al*., 2016). To clarify this platform, industrial engineers need to better understand the influencing factors and then use methods to improve the design tools. In this way, the simulations created by the designers for the workstation design should be closer to the motions made by the workers. Quality improvement methods show great potential in the production process as they use an empirical approach to reduce change and improve work processes (Amaratunga and Dobranowski, 2016). The analysis was originally done using causal diagrams, Pareto charts, study of setup time and performance indexes to find out the main problems, using the tools in lean to improve the quality of production processes such as single-minute exchange of dies (SMED) and 5S. In practice, the application of innovative tools depends on a variety of conditions such as environment, production industry, available resources, etc. Although quality improvement approaches are of many benefits, it is also necessary to conduct research on the risk of the misleading system to better understand and timely issue, timely solutions (Mason *et al*., 2015).

3 METHODOLOGY

The steps are shown in Figure 1 and based on the problems in this company provide more detail on this method.

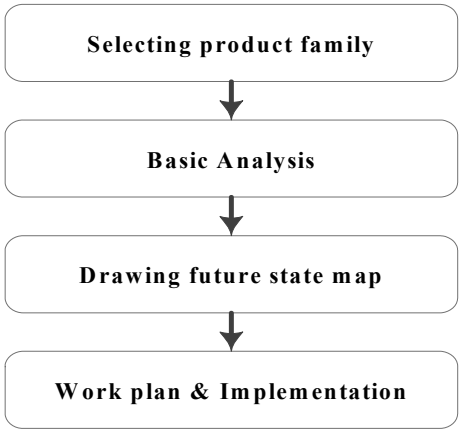

Fig. 1: Methodology of implementation research

3.1 Selecting product family

Applying top-down analysis method, this study has analysed the components, factors related to all operations in this system. To systematic top-down analysis, they need to perform the following tasks such as 1) what are the common goals in the system; 2) collecting and revising all information of each department. This makes discussion and decision easier.

3.2 Basic analysis

Focusing on the information collected, this research paper is to build a logical model that demonstrates relationships between components in the system. From the logical model, using the tools of VSM simulates the operation of parts related to the company's production. Next, simulation results are used to identify the cause and problem of the current system. Based on relevant theory to a system improvement plan developed, the results of the improvement options have been reassessed according to the improved simulation model.

3.3 Drawing future state map

The future state VSM has established based on improved suggestions. In order to minimize inventory wastes, lean tools are used to improve and achieve the capacity of the processes. In the demand stage of this paper, re-designed layout and balanced assembly workstation are applied commonly. The improved actions for the design of a future-status map set up a continuous flow which experiences a process with smoother, without returns, producing in the shortest lead time, highest quality, and lowest production cost.

3.4 Work plan and implementation

The improved suggestions based on the problems have been established, and shown in the next section. The tools employed in this paper make it possible for the organization to get continuous proposal in terms of key stakeholder. All non-value-added activities are systematically and continuously excreted to decrease the costs and rise turnover thanks to lean manufacturing initiatives. Lean plays a vital role in the market for manufacturing industries to survive and succeed. Lean manufacturing includes easy-to-apply and maintainable techniques, and tools that enable organizations to attain planned productivity. Reducing and eliminating wastes gradually become the culture of the organization which might turn every process into revenue.

4 CASE STUDY

4.1 Product family

Currently, the production line of the company always has a large amount of semi-finished products in the field of electronic board production. The layout of the workplace causes many difficulties for

workers with the presence of WIP, especially in the assembly and finished products. Many products have been stored for a long time for packaging and exporting. Therefore, the production of the plant has not been very effective. This article focuses on SMT processing in Figure 2 and has chosen the main product which accounts for 70% of the total products in the company.

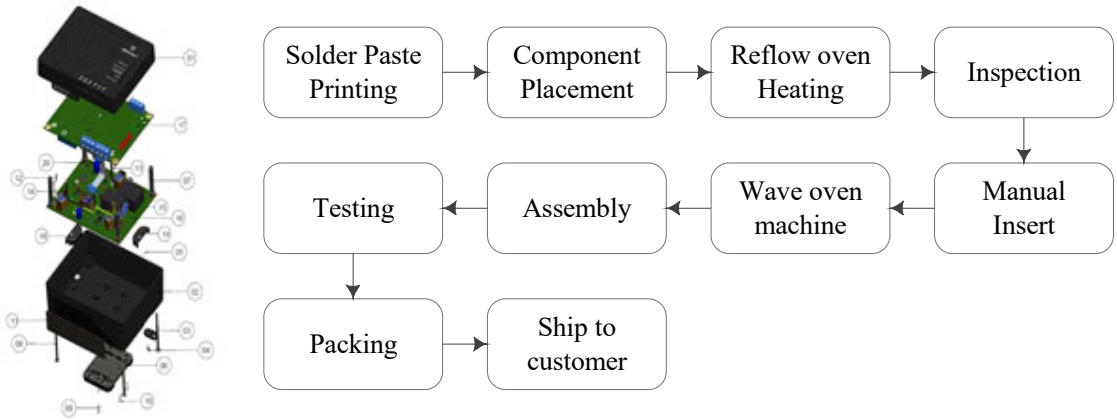

Fig. 2: Processing of the main product SMT line

Surface Mount Technology is an area of electronic assembly used to mount electronic components to the surface of the printed circuit board (PCB) as opposed to inserting components through holes as with conventional assembly. The first machine setup in the manufacturing process is the solder paste printer which is designed to apply solder paste by using a stencil and squeegees to the appropriate pads on the solder paste printing PCB. After pasting, the boards are carried to the pick-and-place machines placing them on a conveyor belt. Each component is picked from its packaging using either a vacuum or gripper nozzle, checked by the vision system and placed in the programed location at high speed. Following the component placement process, it is important to ensure no fault occurrence; moreover, all parts have been correctly placed before reflow soldering. One of challenges for sub-contract manufacturers is the verification of the first assembly to the customer's information or first article inspection which is time-consuming. This is a very important step in the process as any undetected errors, that can lead to high volumes of rework. Once all component placements have been checked, the PCB assembly is moved into the reflow soldering machine where all the electrical solder connections are formed between the components and PCB by heating the assembly to a sufficient temperature. It appears to be less complicated part of the assembly processes; however, the correct reflow profile is key to ensure acceptable solder joints without damaging the parts or assembly due to excessive heat. A carefully-profiled assembly plays a vital role in using lead-free solder since the required reflow temperature need components which may reach maximum rated temperature. Finally, the boards are visually inspected for missing or misaligned components and solder bridging. In case of failure, they are sent to a rework station for repairing. Moisture-sensitive goods in dry bags are also marked with a special label including corresponding warning information

The plastic box is unfocused in this study because it is processed from an external supplier. Customer demand had increased from 10,000 pieces per month in three months (from June to August), but in September the demand increased to 30,000 products per month. Therefore, there are some main points to be taken as 1) the current capacity of the process is not enough to meet customer requirements; 2) the rate of semi-finished products between workstations is high; 3) wastes occurred on the line; 4) resources are inadequate. Working time in the company is 7.58 hours/shift/ day. Data is calculated based on the customer demand. Takt time is 23.7s, the output is relatively hard to produce for 30,000 pieces/month.

– For 10,000 pieces/month:

$$Takt\ Time = Time\ available / Demand\ customer = \frac{7.58 x 26 x 3600}{10000} = 70.92\ (s) \quad (1)$$

– For 30,000 pieces/month:

$$Takt\ Time = Time\ available / Demand\ customer = \frac{7.58 x 26 x 3600}{30000} = 23.7\ (s)$$

Machine workstations are automatically driven by a specified speed, so this study focused on balancing with the manual workstations, which is plug components workstation. Steps for circuit board parts are

passed to component 3 in Figure 2 while work element shown in Table 1 is calculated according to below formula (Niebel and Freivalds, 2003).

Using the sample mean and sample standard deviation: $s = \sqrt{\frac{\sum_{i=1}^{i=n}(x_i-\bar{x})^2}{n-1}}$ (2)

Solving for n yields: $n = \left(\frac{ts}{k\bar{x}}\right)^2$ (3)

Table 1: Observed time in circuit board assembly line

Step	Printed circuit ID	Work Element	s	n	Average Observed time (s)
1	10	Loading pallet & Put Board	1.0	13	13.9
2	20	Insert Capacitor C2	0.7	44	5.3
3	30	Insert Capacitor Thermal Fuse F2&F3	1.3	25	13
4	40	Insert Varistor M1A & M1B	1.5	32	8.4
5	50	Insert Capacitor Thermal Fuse F4&F5	0.2	10	13.8
6	60	Insert M2a, M2b, M2c, M2d,	0.7	91	16.6
7	70	Insert Wire P1	1.4	68	5.5
8	80	Insert Wire P2	1.2	14	4.7
9	90	Insert Relay K1	1.2	14	3.5
10	100	Insert Relay K2	0.8	54	3.9
11	110	Insert P2	0.5	31	4.7
12	120	Insert NCT N1	0.5	26	5.7
13	130	Insert Wire P3	0.5	31	4.7
14	140	Insert M3a, M3b, M4a, M4b2	0.5	31	16.6
15	150	Insert Wire P4	0.5	31	4.7
16	160	Checking component	0.5	31	17.3
17	170	Insert Wire P5	1.4	17	4.7
18	180	Loading pallet & put to machine	1.2	3	36.2
Total					183.2

Before improving (7.58 working hours per day), Standard Time is set up in Table 2 and calculated according to below expression (Niebel and Freivalds, 2003).

- The normal time (NT): NT = OT x R/100 (4)
- The expression for standard time (Becker and Stern): ST = NTx(1+Allowance) (5)

Table 2: SMT line capacity planning

Task No	FC Id.	Station	Basic Cycle			Std. Time (s)	Operator
			Observed time (s)	Rating (%)	Variable Fatigue		
1	10, 20, 30, 40	Manual Insert 1	45.97	90	15	47.58	1
2	50, 60, 70, 80	Manual Insert 2	32.27	90	5	30.49	1
3	90, 100, 110	Manual Insert 3	26.97	90	5	25.48	1
4	120, 130, 140	Manual Insert 4	24.37	90	5	23.03	1
5	150, 160	Manual Insert 5	9.43	90	5	8.91	1
6	170, 180	Manual Insert 6	53.47	90	5	50.53	1
Total						186.02	6

There is no balancing between workstations. Figure 3 shows that the Takt time is 70.92 seconds, but most of the time workers have high idle time; however, the number of required workers are six workers.

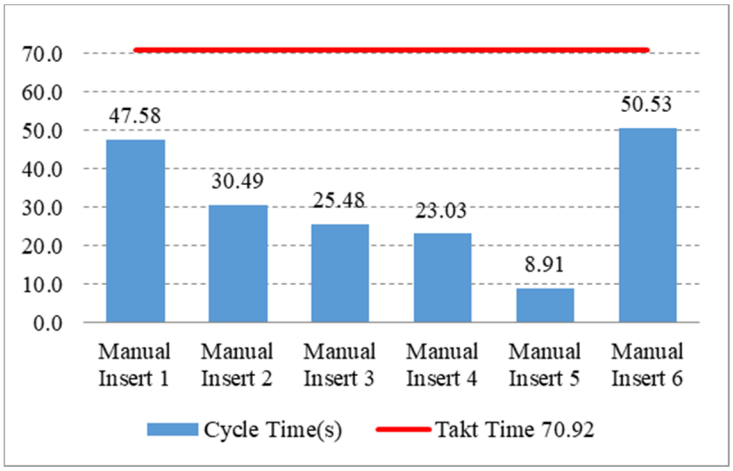

Fig. 3: Cycle time of component 3 processes

4.2 Basic analysis

The current layout in this company is designed in U-shape; therefore, this type of line makes it easy to utilize the most available resources, to balance, to be suitable for lower output, and to change products. Besides, it is difficult to respond to the increasing production because semi-finished products in each stage will increase. After products are finished, they need to be moved to the warehouse area as illustrated in Figure 4

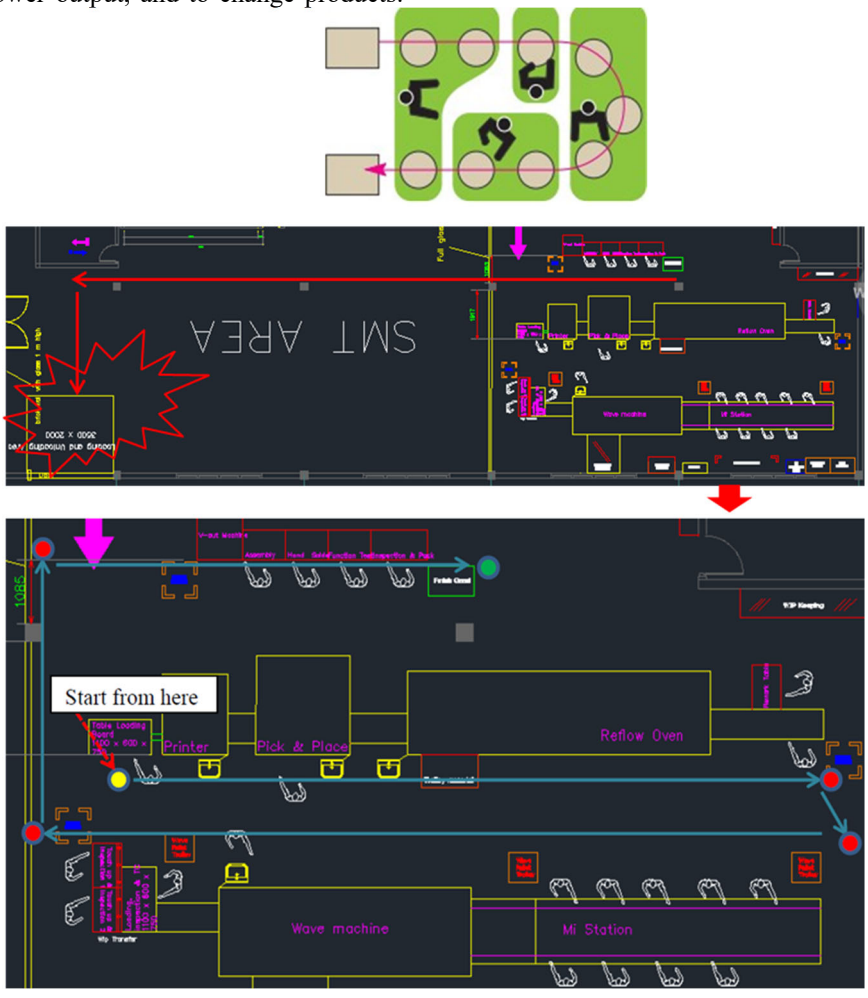

Fig. 4: The current layout of warehouse area and SMT line

The diagram shows some disadvantages which the VSM tool might identify. Moreover, the measured parameters of all stages in this processing in Figure 5 clearly show the disadvantages.

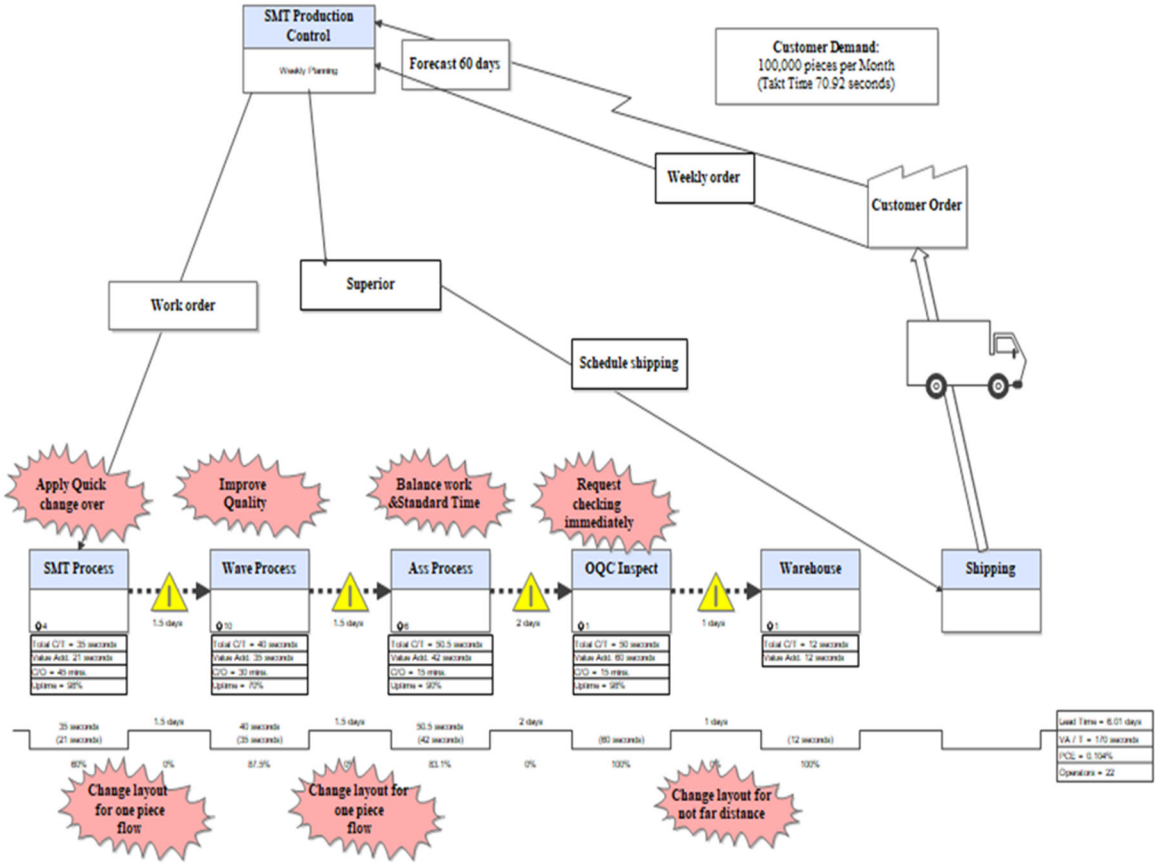

Fig. 5: The current state VSM of SMT line

As shown in Figure 5, it is possible to indicate the types of waste as follow 1) The Waste of Motion: the distance of each station is far, following the Batch to Batch; 2) The Waste of Waiting is unbalanced between the workstations, having many workers to wait for other operations for a long time.

4.3 The future VSM

The company decided to make two shifts each day to meet the customer demand. Working time is as follows: 1) 1st shift (from 6 AM to 2 PM): 30 minutes for breaking time (from 10 AM to 10.30 AM) and 5 minutes of implementing 5S last hour; 2) 2nd shift (from 2 PM to 10 PM): 30 minutes of breaking time (from 6 PM to 6.30 PM) and 5 minutes implementing 5S. Finally, each shift has about 7.42 working hours and 14.84-hr day/2 shifts and meets the demand in 30,000 pieces/month.

$$Takt\ time = Time\ available\ / Demand\ customer = \frac{15x26x3600}{30000} = 46.3\ (s)$$

As shown in Figure 6, productivity increased from 10,000 pieces/month to 30,000 pieces/month (about 50% per a shift), and lead time was reduced from 6 days to 192.76 seconds based on the mentioned solutions in the current VSM. Besides, some solutions have not been performed in this case study such total quality management, total productive maintenance, and SMED that are called long-term solution to improve the working environment.

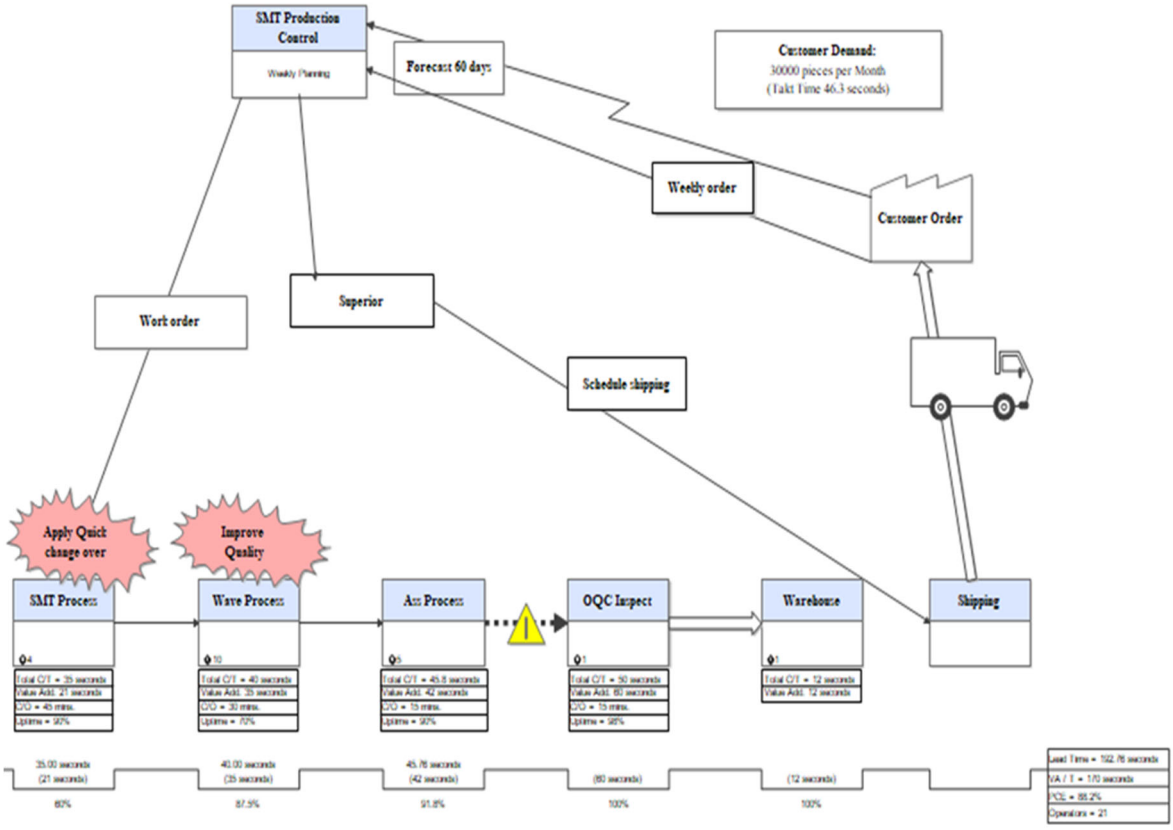

Fig. 6: The future VSM

4.4 Implementation and results

4.4.1 Redesigning layout

There are two main approaches to site relocation problems: building algorithms and improved algorithms. An improved algorithm is used to solve the problem of relocation of premises for this study. Moreover, the cell layout after solving the surface problem has been completed, based on visual perceptions and visual production, which are used to improve and evaluate. The layout is designed as shown in Figure 7 to allow the production flow easily, reducing the rate of semi-finished products at each stage, and decreasing the waste of motion between areas.

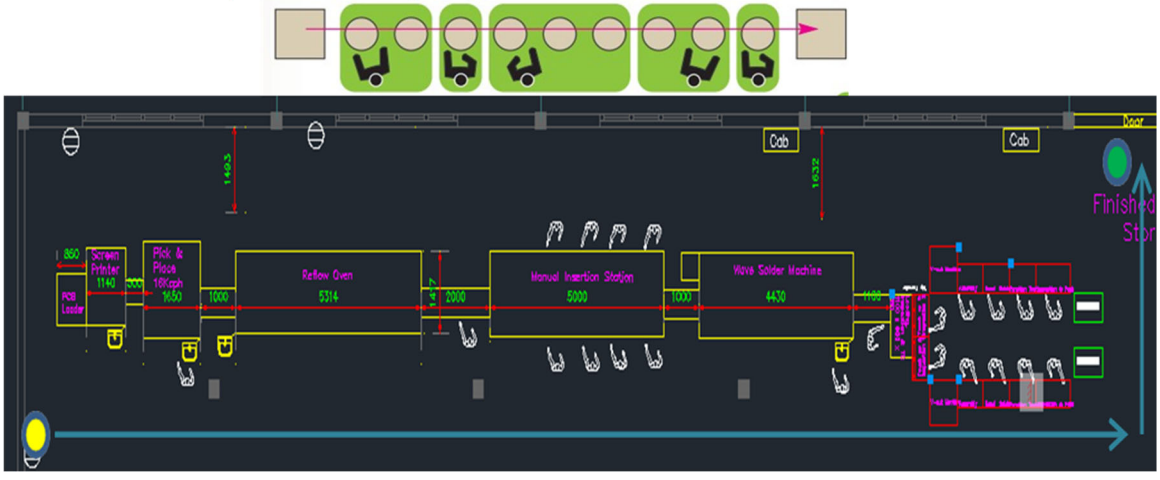

Fig. 7: The redesigned layout in SMT line

4.4.2 Balancing line

It is essential to reduce and improve cycle time to meet better customer demand. Based on data of workers' operations, operations are be arranged more consistently and matched requirement. After planning the new scheduling for 14.8 working hours/ day in assembly workstation, namely setting up the standard time, rating of the performance of Westinghouse system and allowance present in Table 3 (Niebel and Freivalds, 2003).

Table 3: Standard time of assembly WS

Task	FC ld.	Basic Cycle		Allowance (%)		Std. Time (s)	Operator
		Observed time (s)	Rating (%)	Constant	Variable Fatigue		
1	10, 20, 30, 40	40.55	95	9	6	44.30	1
2	50, 60, 70, 80	40.57	95	9	6	44.32	1
3	90, 100, 110, 120, 130, 140	39.02	95	9	6	42.63	1
4	150, 160, 170	26.73	98	9	6	30.12	1
5	180	36.17	110	9	6	45.76	1
Total						207.13	5

In Figure 8, the next proposal might improve cycle time to meet customer demand, especially attending in Manual Insert 4. Basically, rearranging the operations might suit the requirements and the productivity before and after improvements are shown line efficiency is increased from 44% to 89.5%.

$$Line\ efficiency\ (Before) = \frac{Total\ cycle\ time}{Total\ work\ station\ x\ Takt\ Time} = \frac{186.02}{6\ x\ 70.92} = 44\%$$

$$Line\ efficiency\ (After) = \frac{Total\ cycle\ time}{Total\ work\ station\ x\ Takt\ Time} = \frac{207.13}{5\ x\ 46.3} = 89.5\%$$

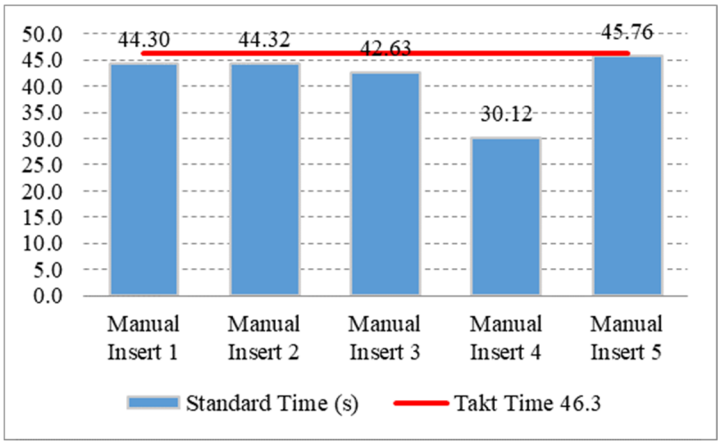

Fig. 8: Compensation of cycle times and Takt time after improving

4.4.3 Improving work environment

Standardized work practices detail how work should be identified the steps and problem operations. Thereafter, the standard is improved according to the given criteria in Figure 9 (Pheasant, 2014)

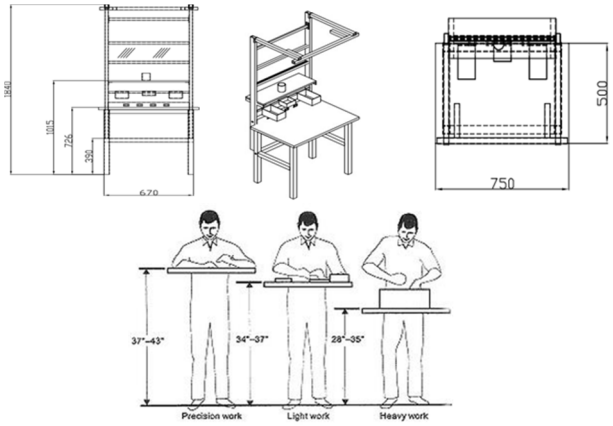

Fig. 9: Designed workstations

All necessary items are arranged in front of the workers, enabling them to work easily. In addition, the lighting system satisfies electronic industry standards, supporting to increase productivity, work efficiency, reducing defects and improve quality. In many cases, workers stand for hours at a hard surface causing fatigue and distraction at work. Fatigue-resistant carpets are offered to solve this problem in Figure 10 (Quinn and Billings, 1989).

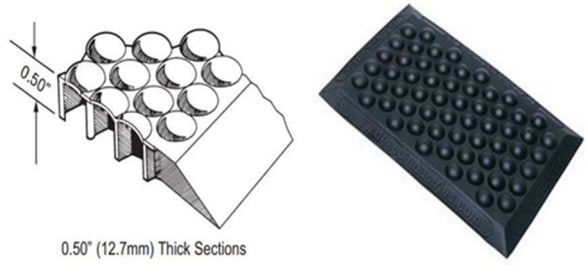

Fig. 10: A sample of fatigue-resistant carpets

Carpets might relieve the impact on the soles of the feet. However, this solution just reduces fatigue of workers while standing for long periods.

4.4.4 Implementing 5S principles

5S application might make the workshop more organized and scientific and save time for research tools. Maintaining a good habit and a clean environment is to improve productivity and quality of work in Figure 11.

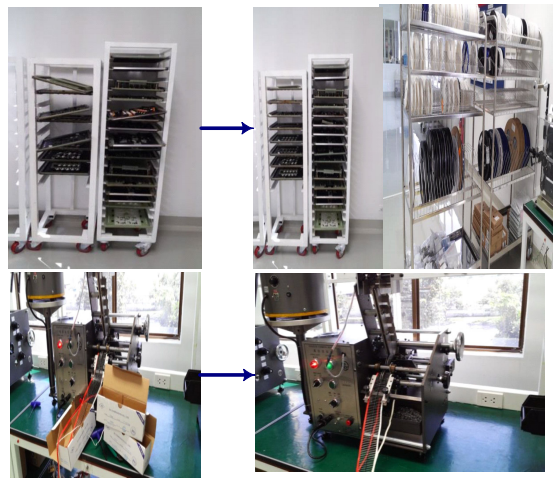

Fig. 11: An implementation of 5S tool

– In addition, it is important to establish standard operation procedure for maintenance guidance and standard time.

– Perform and document preventative maintenance and repair operations follow as required

– Maintenance method follows guided line in the manual book for each model (hard copy or soft copy which latest version).

– Machines have maintenance schedule daily, monthly such as Wave machine, Printer Machine, Reflow machine, Pick and Place machine. Machine maintenance daily performed by SMT technician or Machine Operator and monthly performed by SMT Group (Technician, Engineer). The record is to create a standard operation and follow the instruction after Printer Machine was performed in Figure 12.

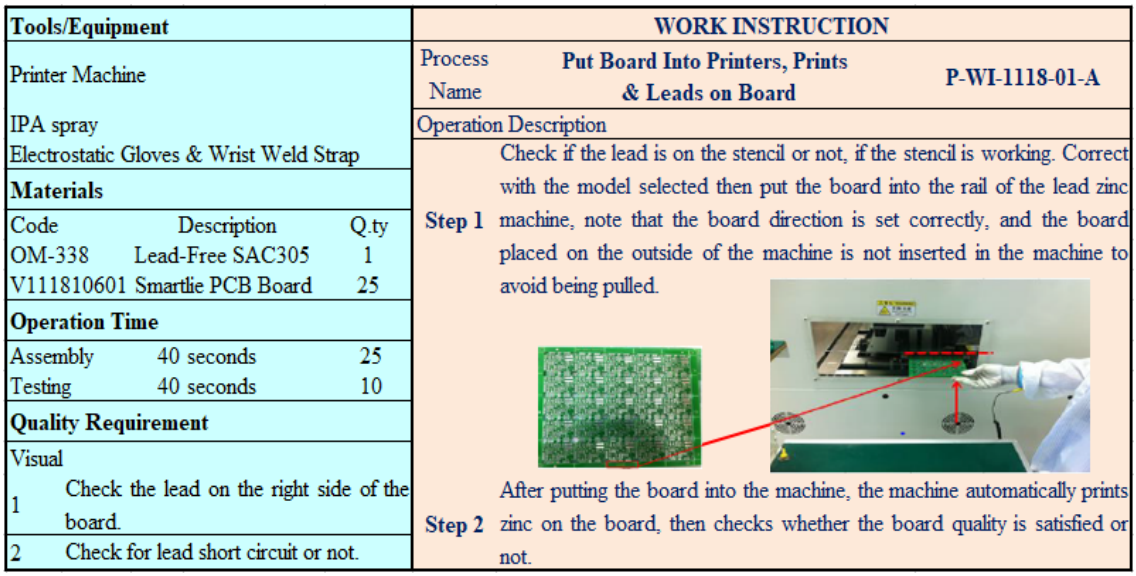

Fig. 12: Standard operation procedure of printer machine

5 RESULTS AND CONCLUSIONS

This paper has met the objectives with the advantages of following strict and scientific methodology, using many tools, achieving the following results after performing lean system such as 1) increasing productivity from 10,000 pieces/month to 30,000 pieces/month (about 50% a shift); 2) reducing lead time from 6 days to 192.76 seconds; 3) improving the proportion of finished products on the line from 44% to 89.5% in Table 4.

Table 4: Comparing the effectiveness of VSM before and after improvement

Content	Before	The future VSM
Lead time	6 days	192.76 s
Operators	22	21
Takt time (second)	70.92	46.3
Line efficiency (%)	44	89.5

Although this implementation has not explicitly shown the saving costs, it helped all staff know about the real meaning of lean system and the activities will be value-added or bring the value back to them. The results of the study are the basis for further research on the application of lean production tools to the production line and the extension of research to other lines in the company as well as other electronics companies.

In general, this research still faces some barriers from workers, being touched by individuals all fresh time. It needs to take a period to change their thinking way individually. Therefore, in order to successfully apply lean model and to maintain efficiency, it is necessary to establish a lean team with a training plan, specific training content, and details clearly for the next research. However, most companies do not realize the majority of production costs in non-value activities. This leads to a bias in the calculation and selection of improved solutions. Therefore, managers need to focus on losses and minimize or eliminate waste by redesigning the future plans which might meet precisely the condition of their company. This will help the company increase its competitiveness in many aspects such as prestige, price, productivity, and quality.

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Genetic diversity of *Pangasius krempfi* in the Mekong River estuaries

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ABSTRACT

Pangasius krempfi is an important catfish species for capture fisheries in the Mekong River basin. Overexploitation could lead to decreasing genetic diversity of this species. This study was aimed to quantify genetic diversity and structure of P. krempfi in the lower Mekong River using ISSR (Inter-simple sequence repeat) markers. Samples were collected from two estuaries of Tien (at Binh Dai, Ben Tre, BT) and Hau Rivers (at Cu Lao Dung, Soc Trang, ST). Twenty individuals per location (or group) were analyzed with six ISSR primers, generated a total of 32 bands with the size ranging from 500 – 2200 bp. The two fish groups had similarly moderate levels of genetic diversity. As the whole population, genetic parameters were (mean ± SE) 56.3 ± 3.1% of polymorphic loci, 1.365 ± 0.048 effective number of alleles, 0.215 ± 0.027 expected heterozygosity, and 0.310 ± 0.037 Shannon index. Genetic distance based on Nei's method between the two groups was 3.4%, accounting for 12% of total genetic variation. Principal coordinate and molecular genetic variance (AMOVA) analyses indicated that the two fish groups were genetically clustered at a low level, suggesting that they can be originated from different spawning groups of the same population.

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1 INTRODUCTION

Pangasius krempfi Fang and Chaux, 1949, a member of Pangasiidae family, distributes along the Mekong River, from Luang Prabang province in northern Laos to coastal areas of Mekong estuaries in Vietnam (Poulsen and Valbo-Jørgensen, 2000; Poulsen and Hortle, 2004; Tran *et al.*, 2013). This is an anadromous species that migrates a long distance from the downstream in the Mekong Delta (Vietnam) to the upstream of the Mekong River (in Laos) for spawning (Baird, 1996; Hogan *et al.*, 2007). *P. krempfi* has high flesh quality and is a preferred food with high economic values. This species is an important fish for capture fisheries in the lower Mekong River basin (Baird, 1996; Poulsen and Hortle, 2004). Because of overexploitation, *P. krempfi* has been listed as vulnerable species (Baird, 2013) and thus needs to be set a high priority for conservation.

In a conservation program, knowledge of genetic diversity and genetic structure of a species is critical (Ellegren *et al.*, 2016). It is well documented that the decline in population sizes causes the loss of genetic diversity, reducing species adaptability to environmental changes in the future (Allendorf *et al.*, 2008; Pinsky and Palumbi, 2014). Up to now, no genetic diversity information of *P. krempfi* is

available. In the Mekong Delta of Vietnam, the species has been found in Hau and Tien Rivers, two branches of the Mekong River (herein fish in two locations are called "fish groups"). Previous studies proposed that *P. krempfi* larvae drift from spawning areas in Khone Fall to the downstream of the Mekong Delta (Baird, 1996; Hogan *et al.*, 2007). If larvae come from the same population and they enter randomly to Hau and Tien Rivers, they should have no genetic difference. Genetic data can be inferred to test this hypothesis.

Different DNA markers can be employed to investigate the genetic diversity of fish species (Liu and Cordes, 2004). Among which, the inter-simple sequence repeat (ISSR) is a dominant marker amplified by a polymerase chain reaction (PCR) with one primer that is complementary to a target microsatellite. Without prior knowledge on DNA of a target species, sequence segments between two neighboring microsatellites are amplified, yielding high polymorphic patterns (Bornet and Branchard, 2001). Thus, the ISSR technique is simple, inexpensive, and effective in population genetics studies.

In the present work, ISSR markers were used to quantify genetic diversity levels of *P. krempfi* in the Mekong Delta and test if two fish groups in Hau and Tien estuaries were genetically similar. Such information is important for better understanding the migratory pathway of the species in the lower Mekong River, contributing to the management and conservation of *P. krempfi*.

2 MATERIALS AND METHODS

2.1 Fish sampling

Fish samples were collected from fishermen at two estuaries at Binh Dai district, Ben Tre (BT) province and Cu Lao Dung district, Soc Trang (ST) province. These sampling sites are located at two branches, Tien branch and Hau branch, respectively, of the Mekong River. The fish was identified based on morphological keys provided from previous studies (Truong Thu Khoa and Tran Thi Thu Huong, 1993; Tran *et al.*, 2013; Duong *et al.*, 2016). Fin clips from 20 samples from each location were used for genetic analysis.

2.2 DNA extraction

DNA was extracted from fin clips using Promega genomic DNA purification kit. First, a piece of fin sample (20 mg) was placed in a 1.5 mL tube with 275 µL of digestion solution (containing 10 mg proteinase K) and then incubated overnight at 55°C. After incubation, 250µl of Wizard® SV Lysis Buffer was added and the entire lysate sample was transferred into a Wizard® SV Mini-column assembly put in a micro-centrifuge tube. The tube was centrifuged at 13,000 × g for three minutes to bind DNA into the Mini-column. Next, the DNA sample was washed four times with 650 µL of Wash Solution (containing 95% ethanol) and centrifuged at 13,000 × g for one minute. Finally, DNA was diluted in TE and stored at –20°C.

2.3 PCR amplification and visualization of ISSRs

Total 29 primers (Table 1) were screened by amplifying two random DNA samples from each sampling location. Primers were chosen based on three criteria including high polymorphisms, reproducibility and visibility on gels. Of the primers screened, six primers were selected for genetic diversity analysis (the first six rows in bold text, Table 1).

Primer amplifications (or PCR) were conducted in a 10 µL mixture containing 5 µL Promega PCR Master Mix (including Taq DNA polymerase supplied in a proprietary reaction buffer (pH 8.5), 400µM dNTPs, and 3mM $MgCl_2$), 0.4 µL primer (10 µM), 1.0 µL DNA, and 3.6 µL nuclease-free water. Thermal conditions for PCRs included one denaturing cycle at 94°C for four minutes, 40 cycles at [94°C for 45 seconds, annealing temperature (*Ta*) from 44°C to 51°C (according to primers) for 45 seconds, extension at 72 °C for two minutes], and one final extension cycle at 72°C for 10 minutes.

PCR products and a 1-kb ladder (Fermatas) were electrophoresed (80 minutes at 50 V) in 1.2% agarose gels. The gels were then soaked in ethidium bromide solution (0.5 µg/mL) before being visualized on a UV- transilluminator. The size of bands was estimated based on the ladder, and then each sample was scored as presence (1) or absence (0) for each band, forming a binary matrix data set for further analysis.

Table 1: List of primers used for screening in the study

No.	Primer	Sequence	Annealing temperature	Reference
1	HB10	5' [GA]6CC 3'	45ºC	Saad *et al.*, 2012
2	ISSR11	5'[CAC]3GC 3'	46ºC	Sharma *et al.*, 2011
3	Chiu-SSR1	5'[GGAC]3A 3'	46ºC	Pazza *et al.*, 2007
4	Chiu-SSR2	5'[GGAC]3C 3'	48ºC	Pazza *et al.*, 2007
5	Micro11	5'[GGAC]4 3'	44ºC	Fernandes *et al.*, 2000
6	EL02B	5'[AG]8T 3'	51ºC	Labastida *et al.*, 2015
7	ISSR03	5'[GACA]4 3'	46ºC	Rout *et al.* 2009
8	ISSR06	5'[GA]8C 3'	46ºC	Labastida *et al.*, 2015
9	ISSR14	5'[GCT]6C 3'	46ºC	Tanhuanpaa *et al.*, 2008
10	ISSR15	5'[TCC]5 3'	46ºC	Raghuwanshi *et al.* 2013
11	EL02A	5'[AG]7C 3'	53ºC	Labastida *et al.*, 2015
12	EL04A	5'AT[GACA]4 3'	53ºC	Labastida *et al.*, 2015
13	EL06A	5'[GACA]4AT 3'	53ºC	Labastida *et al.*, 2015
14	EL03	5'[GTG]5GC 3'	56ºC	Labastida *et al.*, 2015
15	EL05	5'[GAG]5GC 3'	56ºC	Labastida *et al.*, 2015
16	EL06B	5'[GACA]4AC 3'	54ºC	Labastida *et al.*, 2015
17	EL06D	5'[GACA]4TC 3'	54ºC	Labastida *et al.*, 2015
18	17899A	5'[CA]6AG 3'	52ºC	Saad *et al.*, 2012
19	17898A	5'[CA]6AC 3'	48ºC	Saad *et al.*, 2012
20	17898B	5'[CA]6GT 3'	48ºC	Saad *et al.*, 2012
21	844A	5'[CT]8AC 3'	44ºC	Saad *et al.*, 2012
22	844B	5'[CT]8GC 3'	44ºC	Saad *et al.*, 2012
23	841	5'[AG]8T 3'	44ºC	Kumla *et al.*2012
24	ANSSR1	5'[AACC]4 3'	44ºC	Kumla *et al.*2012
25	ANSSR6	5'[GGAT]4 3'	44ºC	Kumla *et al.*2012
26	EL01	5'[AG]8T 3'	52ºC	Labastida *et al.*, 2015
27	HB08	5'[GA]6GG 3'	48ºC	Eshak *et al.*, 2010
28	HB09	5'[GT]6GG 3'	48ºC	Eshak *et al.*, 2010
29	HB11	5'[GT]6CC 3'	48ºC	Eshak *et al.*, 2010

Note: The first six primers in bold text were selected for genetic diversity analysis.

2.4 Data analysis

Genetic diversity parameters including the percentage of polymorphism, private alleles, effective number of alleles, expected heterozygosity, and the Shannon index were estimated for each fish group using GenAlEx 6.5 software (Peakall and Smouse, 2012). Estimates of genetic diversity parameters across loci were compared between the two fish groups using independent-sample T-test in SPSS (version 20.0). Genetic distance and genetic identity between the two fish groups were also evaluated to test whether they are genetically different. To better understand the genetic relationship between Bong Lau fish groups, a group of 10 samples of Tra Ban *Pangasius mekongensis* was used as an outgroup to generate a phylogenic tree. Tra Ban samples were also amplified with the same six ISSR primers as being done for Bong Lau. The phylogenetic tree based on UPGMA (Unweighted pair group method with arithmetic mean) approach was constructed by using programs POPGEN (Yeh *et al.*, 1999) and MEGA 7.0 (Kumar *et al.*, 2016).

3 RESULTS

The amplification of six ISSR primers on 40 fish samples generated a total of 32 allelic bands with the size range from 500 to 2200 bp, in which each primer yielded from 5 to 6 bands. Examples of bands amplified with two primers HB10 and Micro are illustrated in Fig. 2. Genetic diversity of the whole fish samples was moderate, with (Mean ± SE) 56.3 ± 3.1% of polymorphic loci, 1.365 ± 0.048 effective alleles, 0.215 ± 0.027 expected heterozygosity, and 0.310 ± 0.037 Shannon index. Estimates of genetic diversity of fish in BT was insignificantly higher than those of ST fish group (Table 2).

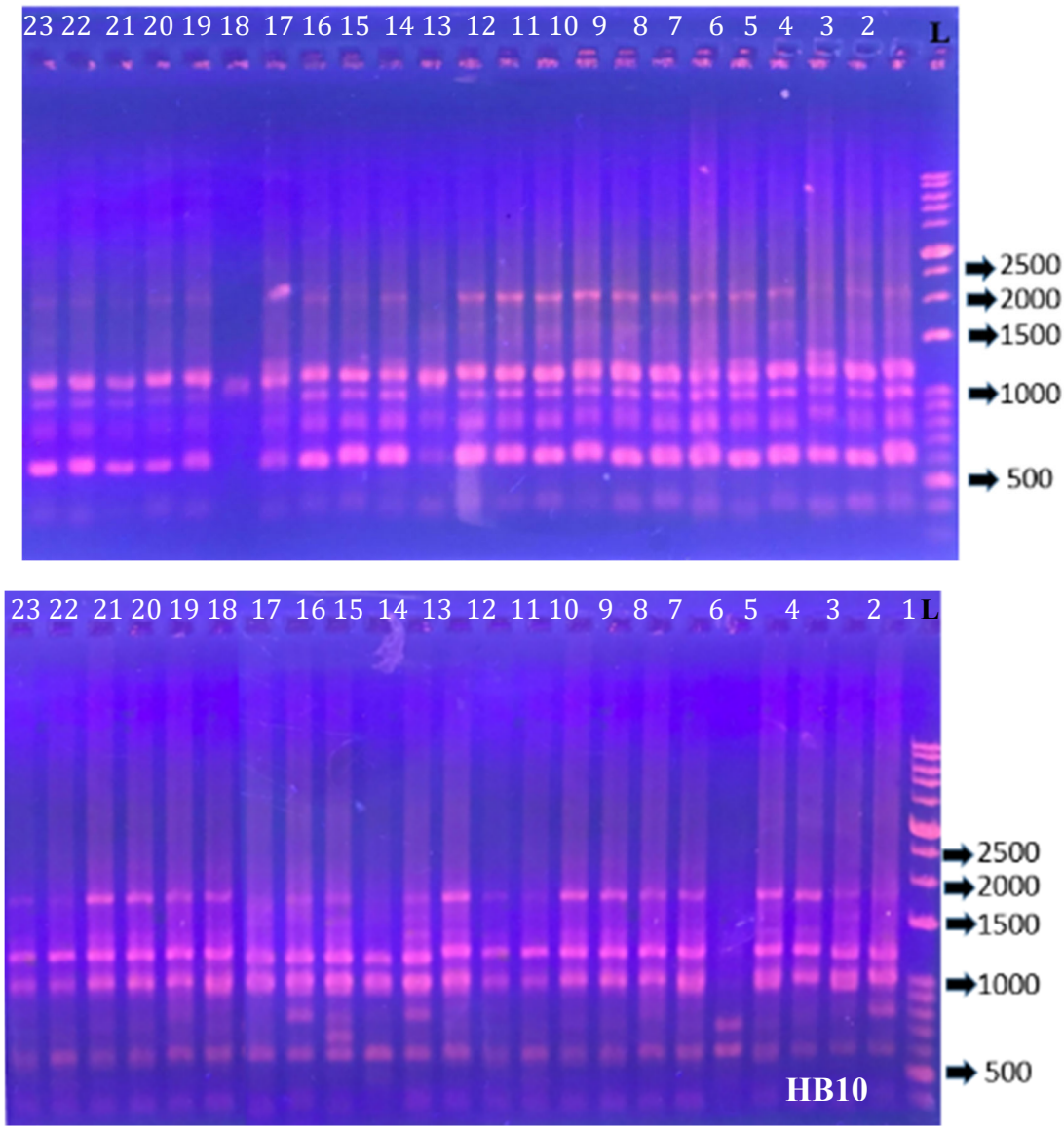

Fig. 2: Examples of ISSR bands from primers Micro11 and HB10 on P. krempfi samples

Note L: ladder; 1 – 12 Ben Tre samples; 13 – 23 Soc Trang samples

Table 2: Genetic diversity indices (SE) of P. krempfi groups produced by ISSR makers

Fish groups	N	Np	%P	Ne	He	I
Ben Tre	20	2	59.4	1.376 (0.069)	0.222 (0.038)	0.320 (0.037)
Soc Trang	20	0	53.1	1.352 (0.069)	0.208 (0.038)	0.299 (0.053)
Total	40	2	56.3 (3.1)	1.365 (0.048)	0.215 0.027)	0.310 (0.037)

Note: N: sample size, Np: private alleles, %P: Percentage of polymorphic loci, Ne: Number of effective alleles, He: Expected heterozygosity, I: Shannon index

Genetic difference between the two fish groups was revealed by Nei's genetic distance, principal coordinates analysis (PCoA), and phylogenetic relationship. Genetic distance between BT and ST groups was 3.4%, or their genetic identity was 96.6%. Analysis of molecular variance (AMOVA) showed that a majority of genetic variation (88%) was from within fish groups, while 12% of genetic variation resulted from between fish groups (Table 3). The PCoA plot (Fig. 3) indicated that the two fish groups were clustered (only a few individuals were mixed

between two fish groups) in the coordinate 1, contributing to 19.1% genetic variation. However, in the presence of Tra Ban data as an outgroup, the two fish groups were more genetically similar and both were distinct from Tra Ban (Fig. 4). The intra-specific genetic distance of Bong Lau was approximately 48-folder less than genetic distance between the two species (estimated based on branch lengths of the phylogenetic tree, Fig. 4).

Table 3: Analysis of molecular variance (AMOVA)

Source	df	Sum of square	Mean of square	Estimated variation	% of total variation
Between fish groups	1	12.1	12.1	0.45	12%
Within fish groups	38	121.9	3.2	3.21	88%
Total	39	134.0		3.66	100%

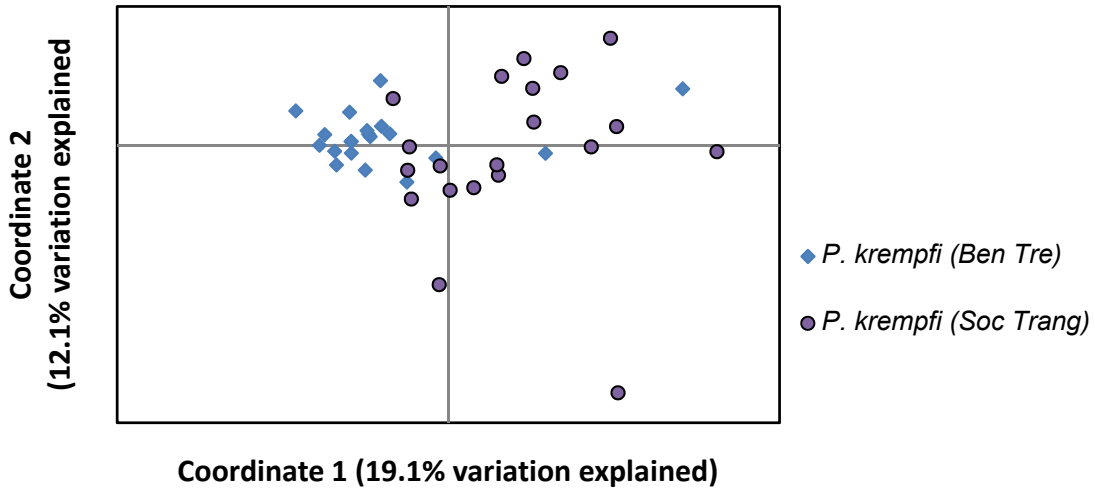

Fig. 3: A plot of principal coordinates analysis (PCoA) of two *P. krempfi* groups

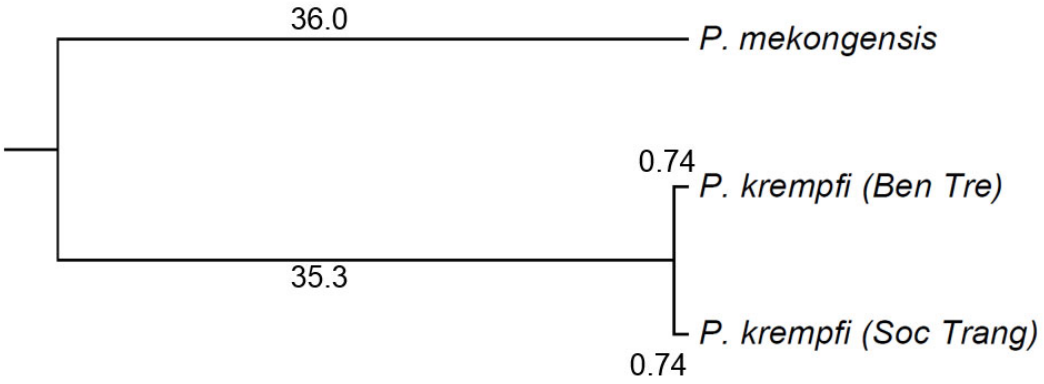

Fig. 4: UPGMA-based phylogenetic tree of *P. krempfi* groups and *P. mekongensis* (numbers present branch lengths estimated based on Nei's genetic distance)

4 DISCUSSION

The results of the present study show that genetic diversity of *P. krempfi* was moderate, and the species had a low level of genetic difference between fish in two branches of the Mekong River. Estimates of genetic diversity parameters of *P. krempfi* were not significantly different between the two fish groups, and these parameters of the whole population (effective alleles Ne: 1.365 ± 0.048, expected heterozygosity He: 0.215 ± 0.027, and Shannon index I: 0.310 ± 0.037) were comparable to those of other species based on similar dominant markers such as ISSR and RADP (Random amplified polymorphic DNA). On climbing perch (*Anabas testudineus*), mean estimates of Ne, He, and I of four wild and cultured populations were 1.364, 0.223, and

0.347, respectively (Pham Thi Trang Nhung and Duong Thuy Yen, 2014). On kissing gourami (*Helostoma temminckii*) in the Mekong Delta, levels of genetic diversity varied among populations, expected heterozygosity ranging from 0.180 to 0.245, and Shannon index from 0.269 to 0.386 (Duong *et al.*, 2018). Another study using ISSR found that lionfish (*Pterois species*) populations in Guanahacabibes (Cuba) had heterozygosity value of 0.253 ± 0.019 (Labastida *et al.*, 2015), relatively higher than that of *P. krempfi*. However, some other species were reported to have lower genetic diversity compared to *P. krempfi*. The yellow catfish (*Mystus nemurus*) populations in Thailand had He (based on seven ISSR markers) from 0.134 to 0.171, and I range of 0.202 to 0.247 (Kumla *et al.*, 2012). In Japanese flounder (*Paralichthys olivaceus*), genetic diversity of three hatchery populations based on 12 ISSR markers was low with He from 0.092 to 0.108, and I from 0.117 to 0.143 (Liu *et al.*, 2006).

Principal coordinates analysis (variation showed by coordinate 2, Fig. 3) and between-group genetic variation (12% of total variance, Table 3) indicate that two groups of *P. krempfi* had clustering structure at a low level. Their genetic distance (3.4%) was lower than that of inter-populations (based on ISSR markers) in other species. For example, genetic distances among populations of yellow catfish in Japan were high, from 14.9% to 61.9% and correlated with geographic distance, indicating genetic isolation by distance of these populations (Kumla *et al.*, 2012). Genetic distance among four kissing gourami populations in the Mekong Delta was from 2.3% to 10.2 (Duong *et al.*, 2018), comparable to or higher than that of *P. krempfi*.

Low genetic distance and weak genetic structure of *P. krempfi* groups indicated that they originate from the same population. This result can be explained by migration behavior of the species and water connectivity in Mekong estuaries. *P. krempfi* is anadromous, the adult fish migrates upstream of the Mekong River (in Laos) for spawning (Baird, 1996; Hogan *et al.*, 2007). When young fish individuals drift downstream to southern Vietnam, they can enter two branches (Tien and Hau Rivers) of the Mekong River. Thus, fish samples collected from different downstream locations can be originated from the same population. In addition, in estuary areas, *P. krempfi* can migrate along the coastal line including two sampling sites in Hau and Tien estuaries.

However, the two fish groups exhibited a small level of genetic structure (Table 3 and Fig. 3), probably because young fish in two locations could be produced by different broodstock groups spawning at different times of the same population. The spawning season of *P. krempfi* can be from May to early November when this species has been observed to be in maturation conditions at Khone Falls in Laos (Baird, 1996). Such a long spawning season is more likely attributed by several spawning groups returning to the spawning ground at different times. Several studies in other migratory species such as pink salmon *Oncorhynchus gorbuscha* (Smoker *et al.*, 1998) or lake sturgeon *Acipenser fulvescens* (Forsythe *et al.*, 2012) found that difference in spawning time had a genetic basis. Consequently, spawning groups can be genetically different. Coulson *et al.* (2006) found that early and late spawning adults of rainbow smelt *Osmerus mordax* along the east coast of Canada had genetic differentiation with the magnitude of difference comparable with that of spatially separated populations. Similarly, *Prochilodus costatus*, a freshwater migratory fish, also displays genetic structure among adult groups within a spawning season (Braga-Silva and Galetti, 2016). Based on the result of the present study, a hypothesis is proposed that spawning adults of *P. krempfi* can consist of several groups differing in spawning time and/or genetics. This hypothesis is similar to a prediction from previous studies (Rainboth, 1996; Sokheng *et al.*, 1999). These authors predicted that there may be at least two spawning populations of *P. krempfi*, one population in the upper Khone Falls migrates for spawning from May to September; the other population in the lower Khone Falls spawn between May and August. Further ecological and genetic studies in upstream sites (in Laos) can test this hypothesis.

5 CONCLUSION AND RECOMMENDATION

P. krempfi in the downstream of the Mekong River has moderate genetic diversity and low genetic structure inferred from ISSR markers. The results suggest that the two fish groups can be originated from different spawning groups of the same population in the upstream.

The species should be concerned under proper management strategies. In addition, more ecological and genetic information of this species in the upstream sites should be investigated for conservation purposes of this species.

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Growth and survival rate of mud clam larvae (*Geloina* sp.) in relation to rearing densities and diets

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ABSTRACT

Different stocking densities and feed types were evaluated in rearing larvae of mud clam Geloina sp. The experiment 1 included four feed treatments as (i) 100% fresh algae (Nannochloropsis sp and Chaetoceros sp with 1:1 ratio), (ii) 100% dry algae (Spirulina powder), (iii) 75% fresh algae + 25% dry algae, and (iv) 50% fresh algae +50% dry algae. Mud clam larvae were cultured in 5L bottles at the stocking density of 2,000 larvae/L. Experiment period was lasted 17 days from D-larvae to umbo stage (from planktonic to benthic stage). Experiment 2 included three treatments with different nursing densities of clam larvae from (i) 2,000 larvae/L, (ii) 4,000 larvae/L to (iii) 8,000 larvae/L. The best feed type from experiment 1 was applied for feeding in experiment 2. Results from experiment 1 showed that in treatment with 100% fresh algae, the length and width of larvae reached highest values (217.3µm and 230.0 µm) on day 17. In this treatment, metamorphosis rate (31.2%), survival rate (10.5%) and larvae production (300 ind./L) also reached higher values than in other treatments ($p<0.05$). In experiment 2, the best result was obtained when nursing clam larvae at 2,000 ind./L. Length and width of larvae reach highest values (218.3µm and 231.3µm on day 17, respectively), metamorphosis rate (34.9%), survival rate (10.3%) and larvae production (294 ind./L) were also highest in this treatment.

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1 INTRODUCTION

Mud clam (*Geloina* sp.) is one of the edible bivalve species. This genus is one of the largest mangrove bivalves, growing to maximum shell length of 8 cm. The utilization of bivalve species becomes popular due to its high protein content and deliciousness (Ismail, 2015).

Several studies have been conducted on this species and mainly focused on characteristics of reproduction (Morton, 1985) or distribution and morphology (Gimin *et al*., 2005). In Vietnam, several studies have been carried out to culture mud clam in shrimp pond or integrated culture with shrimp and mud crab (Nguyen Van Trai, 2015). With the study on reproductive cycle, Quach Kha Ly and Ngo Thi Thu Thao (2011) reported the spawning activities of mud clam occurred year around with the peaks in May and November in mangrove system of Ca Mau province. Recently,

Ngo Thi Thu Thao *et al*. (2018) also reported the induced methods that could improve the spawning efficiency of mud clam from U Minh Thuong, Kien Giang province.

Nowadays, mud clams have been cultured with different systems; however, mostly seed sources are collected from the wild. Therefore, with limited numbers it can not fulfill the requirements for the culture in large scale. Methods to induce spawning of mud clams in hatchery were also recommended by Quach Kha Ly and Ngo Thi Thu Thao (2011), and Ngo Thi Thu Thao *et al*. (2018). However, the techniques for larval nursing or spat rearing for this species are still very limited. It is necessary to find out suitable techniques in order to improve survival rates and growth performance of mud clam larvae in the hatchery. This study was aimed to find out the suitable density and practical diet for nursing mud clam larvae in hatchery conditions. The results from this study will contribute initial information for artificial seed production process of mud clam in Vietnam, especially in the Mekong Delta.

2 MATERIALS AND METHODS

2.1 Clam broodstocks

Matured clam broodstocks with shell length ≥40 mm were collected from Kien Giang province and transported to the hatchery in Can Tho University. The gonad development of bloodstocks had to be at stage III or IV of development. At the hatchery, broodstocks were cultured for 2-4 days in composite tanks with aeration and the salinity at 10 ppt to recover their health after transportation. Then the thermal shock method was applied to induce clam spawning and to collect larvae for experiment.

2.2 Experimental design

2.2.1 Effects of different feed types in rearing mud clam larvae

Sea water was filtered by using filter bag, then treated with chlorine at the concentration of 30 ppm in 2-3 days with strong aeration. EDTA (ethylene diamine tetracetic acid) was added at the concentration of 10 mg/L to neutralize heavy metals in rearing sea water. Salinity was maintained at 10 ppt during experimental period. After broodstock spawning, fertilized eggs were collected and incubated for 24 hours until reach D-larvae stage and then transferred into 5L-glass bottles with initial stocking at 2,000 larvae/L.

Experiment 1 was completely randomized design with three replications of four feeding treatments including i) 100% fresh algae (control, FA); ii) 100% dry algae (DA); iii) 75% fresh algae + 25% dry algae (3FA:1DA); and iv) 50% fresh algae + 50% dry algae (FA: DA). In control group, clam larvae were fed with the mixture of fresh algae species of *Nannochloropsis* sp. and *Chaetoceros* sp. at the cell ratio of 1:1, at the density of 5,000 cells/mL with two feeding times a day at 8:00 and 18:00. Previous study confirmed that the diet with two algal species resulted in better growth and survival rate of white clam, *Meretrix lyrata* (Nguyen Dinh Hung *et al*., 2003). The amount of dried algae in other treatments for daily feeding was calculated based-on the dry cell weight of the live algae in the control diet.

In first five days of culturing, the feces or death algae at the bottom of culture medium were removed daily by siphoning, and new sea water were added to maintain the water volume. Experiment was set up indoor conditions with light intensity around 1,000 lux at day time and darkness at night time. Experiment period was lasted 17 days from D-larvae to umbo stage and transferring into clam spat.

2.2.2 Effects of different densities in rearing mud clam larvae

Experiment 2 was completely randomized design with three replications of three treatments including i) 2,000 larvae/L; ii) 4,000 larvae/L; and iii) 8,000 larvae/L.

The best diet from Experiment 1 was applied for feeding in Experiment 2. The feeding amount and schedule, experiment period and management were similar to those in Experiment 1.

2.3 Data collection

2.3.1 Water quality parameters

Temperature in each culture bottle was recorded daily at 7:00 and 14:00. pH values were examined by pH meter (HANA), concentrations of NH_4^+ and NO_2^- were measured at day 1, 3, 6, 9, 12, 15 and 17 of culture period by SERA test (Made in Germany).

2.3.2 Growth, metamorphosis and survival of larvae

Larvae were collected at day 1, 3, 6, 9, 12, 15 and 17 to measure the shell length and width (µm). At each sampling time, 10-20 larvae in each glass bottle were collected and stained with lugol solution. Shell length and width of each larvae was measured under microscope with micro-ruler.

Metamorphosis rate (%) of larvae from D-stage to Umbo stage was determined from day 9 to day 17 of rearing period and will be calculated as follows:

Metamorphosis rate (%) = 100 × (Number of Umbo larvae/ Number of observed larvae).

To record the survival rate, larvae in rearing bottle was collected at four points around and one point at the middle of bottle with the volume of 5 mL. Then, they were filtered through suitable mesh size. A number of alive larvae were counted by Sedgwick Rafter counter and calculated the survival rate.

Survival rate (%) = 100 × (alive larvae/initial number of larvae at the beginning of experiment)

2.3.3 Data analysis

One-way ANOVA followed by Duncan test was applied to detect the significant difference of collected parameters among treatments at $p<0.05$ using SPSS program version 22.0.

3 RESULTS

3.1 Effect of different diets on growth and survival rate of mud clam larvae

3.1.1 Variation of environmental conditions

Mean temperature was from 26.4 to 27.3°C in the morning and from 28.4 to 28.5°C in the afternoon. pH values also varied slightly in the morning (8.2-8.3) and in the afternoon (8.4-8.5). However, the concentrations of NH_4^+ and NO_2^- were changed among treatments (from 0.2 - 0.5 mg/L). There was no significant difference in temperature or pH values among treatments; however, the concentrations of NH_4^+ and NO_2^- were high in fully dry algae feeding treatment (Table 1).

Table 1: Variation of environmental conditions in different treatments

Treatment	Temperature (°C)		pH		NH_4^+ (mg/L)		NO_2^-(mg/L)	
	7:00	14:00	7:00	14:00	7:00	14:00	7:00	14:00
FA	26.4±0.2	28.5±0.1	8.2±0.1	8.5±0.1	0.2±0.1	0.2±0.1	0.2±0.1	0.2±0.1
DA	27.3±0.1	28.4±0.1	8.3±0.1	8.4±0.1	0.5±0.4	0.5±0.4	0.5±0.4	0.5±0.4
3FA:1DA	27.3±0.1	28.4±0.2	8.3±0.1	8.5±0.1	0.2±0.1	0.2±0.1	0.2±0.1	0.2±0.1
FA:DA	27.3±0.1	28.4±0.1	8.3±0.1	8.5±0.1	0.2±0.1	0.2±0.1	0.2±0.1	0.2±0.1

3.1.2 Shell length and shell width of clam larvae

Shell length of larvae in FA (100% fresh algae) was always longer than that in other treatments (Table 2). On day 17, shell length of larvae was highest in FA treatment (217.3 µm), whereas these numbers were lower in 2FA:1DA (199 µm), FA: DA (198.6 µm) and DA (167.9 µm). There was no significant difference in shell length of larvae between 3FA:1DA and FA:DA treatment on day 17 ($p>0.05$).

Table 2: Shell length and width of larvae (µm) in different treatments

Days	Diets			
	FA	3FA:1DA	FA:DA	DA
Shell length (µm)				
1	133.3±0.6^b	128.1±0.6^a	127.0±0.7^a	127.3±0.8^a
3	136.2±0.6^c	134.1±0.8^b	133.2±0.4^b	130.1±0.3^a
6	141.3±3.2^c	138.5±0.3^{ab}	137.9±0.6^b	134.6±0.6^a
9	147.8±2.1^b	142.1±0.6^a	141.9±0.8^a	144.5±5.1^{ab}
12	151.0±3.5^b	146.2±1.0^a	146.3±1.0^a	145.7±2.1^a
15	165.2±5.7^b	158.8±0.8^a	159.0±0.7^a	155.2±2.1^a
17	217.3±0.6^c	199.0±0.4^b	198.6±1.0^b	167.9±0.4^a
Shell width (µm)				
1	142.4±1.4^c	136.5±0.3^b	135.9±0.4^b	132.3±2.7^a
3	145.9±1.4^c	140.3±0.1^b	139.0±0.9^b	134.3±0.9^a
6	149.2±1.9^c	145.3±0.5^b	143.4±1.5^b	139.8±0.4^a
9	153.2±4.8^b	145.6±0.3^a	145.5±0.9^a	144.4±0.8^a
12	158.6±1.7^b	153.3±0.4^a	153.3±0.4^a	153.3±2.1^a
15	174.3±5.5^c	168.4±0.1^b	168.0±0.3^b	162.1±1.5^a
17	230.0±0.1^c	205.8±0.8^b	203.3±2.9^b	174.8±0.4^a

Values of the same row with the same letters are not significantly different ($p>0.05$)

On day 17, shell width of larvae was also highest in FA (230 µm), whereas it was 205.8 µm, 203.3 µm and 174.8 µm in 3FA:DA and FA:DA, DA treatments, respectivively. Shell width of larvae in 3FA:DA and FA treatment was also not significantly different on day 17 ($p>0.05$).

3.1.3 Metamorphosis and survival rate of mud clam larvae

Clam larvae began to metamorphose at day 9 (Table 3). Metamorphosis rates of larvae on days 9, 12, 15 and 17 in FA treatment were 7.5, 8.9, 26.6 and 31.2%, respectively and were higher than in other treatments ($p<0.05$).

Table 3: Metamorphosis and survival rate of mud clam larvae

Treatment	Rearing day						
	1	3	6	9	12	15	17
Metamorphosis rate (%)							
FA	0.0	0.0	0.0	7.5±0.1^{d}	8.9±0.6^{d}	26.6±1.2^{d}	31.2±0.6^{d}
3FA:1DA	0.0	0.0	0.0	4.3±0.2^{c}	7.4±0.3^{c}	21.9±0.2^{c}	26.2±0.4^{c}
FA:DA	0.0	0.0	0.0	3.4±0.1^{b}	6.6±0.3^{b}	18.9±0.6^{b}	22.5±0.1^{b}
DA	0.0	0.0	0.0	2.3±0.1^{a}	5.8±0.3^{a}	15.8±0.7^{a}	18.8±0.6^{a}
Survival rate (%)							
FA	100^{a}	56.7±0.8^{d}	31.0±0.6^{d}	21.8±0.7^{d}	16.8±0.7^{d}	15.1±0.4^{d}	10.5±0.6^{d}
3FA:1DA	100^{a}	42.7±0.6^{c}	24.1±0.3^{c}	18.2±0.7^{c}	12.8±0.4^{c}	11.0±0.6^{c}	5.8±0.6^{c}
FA:DA	100a	34.9±0.9^{b}	19.4±0.5^{b}	14.3±0.3^{b}	10.9±0.4^{b}	8.4±0.2^{b}	4.5±0.6^{b}
DA	100^{a}	29.4±0.8^{a}	14.9±1.0^{a}	10.3±0.6^{a}	9.0±0.4^{a}	6.4±0.3^{a}	3.9±0.6^{a}

Values of the same column with the same letters are not significantly different ($p>0.05$)

Survival rate of larvae in all treatments decreased abruptly from day 3 to 17 (Table 3). At day 3, 6, 9, 12, 15 and 17, the survival rate of larvae in FA treatment was highest and significantly different from others ($p<0.05$). Especially, at day 17, survival rate was 10.5% in FA treatment and double times higher than in 3FA: DA (5.8%) and FA: DA (4.5%). In treatment fed 100% dry algae, the survival rate was lowest at day 17 (3.9%).

3.2 Effects of different rearing densities on growth and survival rate of clam larvae

3.2.1 Environmental conditions

Mean temperature in rearing medium varied from 26.4-26.5°C in the morning and 28.5°C in the afternoon. pH values varied from 8.2- 8.5, and mean concentrations of NH_4^+ (0.2 mg/L) or NO_2^- (0.2 mg/L) were stable and not significantly different among treatments (Table 5).

Table 5: Variation of environmental conditions in different stocking densities

Density	Temperature (°C)		pH		NH_4^+ (mg/L)		NO_2^- (mg/L)	
(larvae/L)	7:00	14:00	7:00	14:00	7:00	14:00	7:00	14:00
2000	26.4±0.2	28.5±0.1	8.2±0.1	8.5±0.1	0.2±0.1	0.2±0.1	0.2±0.1	0.2±0.1
4000	26.5±0.2	28.5±0.1	8.2±0.1	8.5±0.1	0.2±0.1	0.2±0.1	0.2±0.1	0.2±0.1
8000	26.4±0.2	28.5±0.1	8.3±0.1	8.5±0.1	0.2±0.1	0.2±0.1	0.2±0.1	0.2±0.1

3.2.2 Shell length and shell width of clam larvae at different rearing densities

Shell length of larvae has no significant differences among treatments ($p>0.05$) on day 1, 3, 6 and 9. However, the significant differences were detected at day 12, 15 and 17 of rearing period ($p<0.05$), showing the effects of density on shell growth of clam larvae (Table 6). On day 17, shell length of larvae was highest in lowest rearing density (218.3 µm) and significantly higher than in the remaining treatments ($p<0.05$). As shown in Table 6, shell width of clam larvae was affected by rearing density earlier than shell length. From day 6 to 17, the highest number was always presented in rearing density of 2,000 larvae/L, meanwhile it was similar between middle or highest density.

Table 6: Shell length and width of larvae (µm) in different rearing densities

Day	Stocking densities (larvae/L)		
	1) 2,000	2) 4,000	3) 8,000
Shell length of larvae (µm)			
1	133.3±0.6[a]	133.3±0.6[a]	133.3±0.6[a]
3	136.2±0.6[a]	136.2±0.6[a]	136.2±0.6[a]
6	141.3±2.8[a]	141.3±3.2[a]	140.1±0.7[a]
9	147.5±2,0[a]	143.8±5.4[a]	141,0±0.9[a]
12	151.5±3.6[b]	145.8±4.5[a]	143.7±0.8[a]
15	168.8±0.6[b]	164.9±5.4[b]	158.2±0.6[a]
17	218.3±0.3[b]	208.3±2.9[a]	206.7±2.9[a]
Shell width of larvae (µm)			
1	143.5±0.4[a]	143.5±0.4[a]	143.5±0.4[a]
3	146.5±1.0[a]	145.1±0.7[a]	141.8±1.5[a]
6	149.7±1.6[b]	147.9±0.8[ab]	146.1±0.7[a]
9	155.9±2.6[b]	153.0±0.4[ab]	150.7±0.1[a]
12	158.1±2.5[b]	153.8±2.0[a]	151.4±0.6[a]
15	175.6±1.1[b]	171.2±3.2[a]	169.1±1.0[a]
17	231.3±1.1[b]	206.8±2.8[a]	206.7±2.9[a]

Values of the same row with the same letters are not significantly different (p>0.05)

3.2.3 Metamorphosis and survival rate of mud clam larvae

In Experiment 2, metamorphosis rate of larvae also occurred at day 9 of rearing. Metamorphosis rate at day 9, 12, 15 and especially day 17 (34.9%) was highest in treatment 1 and significantly different from treatment 2 and 3 ($p<0.05$).

Survival rate of larvae strongly decreased from day 3 to day 17 (Table 9) in all treatments. On day 3, 6, 9, 12, 15 and 17, survival rate was highest in treatment 1, and there were statistically significant differences between treatment 1 and 2 ($p<0.05$). At the end of rearing period, clam larvae in lowest stocking density showed the best survival rate (10.3%), meanwhile, corresponding numbers were low in middle (6.6%) and highest stocking density (6.4%). Regardless of double times rearing density, there were no significant differences in survival rate of larvae between density of 4,000 and 8,000 larvae/L ($p>0.05$).

Table 9: Metamorphosis and survival rate of mud clam larvae in different rearing densities

Density (larvae/L)	Rearing days						
	1	3	6	9	12	15	17
Metamorphosis rate (%)							
1) 2000	0	0	0	4.4±0.1[b]	6.6±0.1[b]	21.5±0.3[b]	34.9±0.6[b]
2) 4000	0	0	0	2.3±0.1[a]	4.2±0.1[a]	14.2±0.1[a]	22.9±0.3[a]
3) 8000	0	0	0	2.3±0.1[a]	4.2±0.2[a]	13.9±0.2[a]	22.4±0.2[a]
Survival rate (%)							
1) 2000	100[a]	59.4±0.5[b]	35.1±0.4[b]	29.5±0.6[b]	18.9±0.4[b]	17.0±0.4[b]	10.3±0.2[b]
2) 4000	100[a]	55.0±0.8[a]	33.1±0.5[a]	19.2±0.7[a]	13.4±0.2[a]	10.6±0.4[a]	6.6±0.1[a]
3) 8000	100[a]	55.0±0.6[a]	32.9±0.5[a]	19.5±1.3[a]	13.5±0.3[a]	10.5±0.2[a]	6.4±0.2[a]

Values of the same column with the same letters are not significantly different (p>0.05)

4 DISCUSSIONS

4.1 Effects of different diets on growth and survival rate of mud clam larvae

Richard *et al*. (2015) reported that temperature has a strong influence on Manila clam immune activity. However, in both experiments from this study the temperature was not high variation among treatments, and there was probably no effect on the growth of clam larvae. On the other hand, in experiment 1, the concentration of nitrite was highest in treatment fed dry algae, and it might be harmful for larvae during nursing period.

The findings from Experiment 1 was similar to the results of Albentosa *et al*. (1997) that the growth rates of *Ruditapes decussatus* larvae fed dried microalgae were significantly lower than those from fresh algae diets. The cell size of *Nannochloropsis*

sp. and *Chaetoceros* sp. is suitable for D-larvae. The content of lipids, proteins (amino acids), carbohydrates and vitamins of various micro algae species is one of the main reasons for considering these organisms as food source for aquaculture animals (Southgate, 2003). Furthermore, their content of highly unsaturated fatty acids, especially eicosapentaenoic acid, arachidonic acid, docosahexaenoic acid and linolenic acid provides the most prominent determinant of the nutritional composition of microalgae (Lavens and Sorgeloos, 1996; Richmond, 2004; Martínez-Fernández *et al*., 2006). Becker (2004) suggested that the larval stage needs a small amount but requires a high quality of microalgae.

The partial replacement of a diet of fresh microalgae has been reported in several studies using dried diets. Doroudi *et al*. (2002) reported that black-lip pearl oyster *Pinctada margaritifera* with the substitution of 25–50% of a live microalgae diet with dried micro-algae (*Tetraselmis* sp) did not significantly reduce survival or growth of D-stage larvae. However, results from the present study showed that dried diet substitution negatively affected growth of larvae, with an increased substitution level resulting in growth decline in general. The slowest growth occurred at complete replacement of fresh algae. Dry powder of *Spirulina* algae easily made pollution in cultured environment and triggered bacterial contamination. Furthermore, with the high protein concentration (~50%), the decomposition of *Spirulina* powder will increase nitrite concentrations in nursing water, and it will be harmful for clam larvae. Laramore (2015) recommended that the concentration of nitrite during clam nursing stage should be lower than 0.2 mg/L. The results from this study showed that, dry algae should be limited utilization when fresh algae are in shortage production for rearing D-larvae of mud clam.

Survival rate of clam larvae decreased gradually along the experiment period, at the late development stages, the survival rate of larvae decreased abruptly because they completed the development and started to transfer from planktonic to benthic stage. Quayle and Newkirk (1989) recommended that supplying food sources with micro algal diets at enough quantity and quality played important role in maintaining the good growth performance, high and stable survival rate of larvae in the hatchery.

4.2 Effects of different rearing densities on growth and survival rate of mud clam larvae

Siphoning waste materials from the bottom of cultured vessels and renewing water were conducted daily during experiment, resulting in that NH_4^+ and NO_2 concentrations were not different among treatments of 4,000 and 8,000 larvae/L. Therefore, those toxic nitrogen compounds could be limited effects on the growth of mud clam larvae in this study. The results indicated that growth rate, metamorphose and survival rate were highest at the density of 2,000 larvae/L. Similarly, according to La Xuan Thao *et al*. (2004), cultured blood cockle larvae at 25 ppt and the density of 2,000 individuals/L were the optimum conditions for nursing blood cockle in planktonic stage. The authors also observed that the growth rate and survival rate of blood cockle larvae were decreased when nursing densities increased from 2,000 to 4,000 larvae/L.

The growth of larvae in high rearing density remained relatively low, supporting the notion that factors other than access to food limit growth at high stocking density (Liu *et al*., 2006). Elevated density triggers a corresponding increase in the collision rate between swimming larvae causing retraction of the velum and cessation of feeding, as well as longer term energy loss associated with shell repair (Cragg, 1980; Sprung, 1984; Liu *et al*., 2006). Higher larval density may also result in rapid accumulation of toxic metabolites that can be detrimental to both immune function and growth (Sprung, 1984; Yan *et al*., 2006; Raghavan and Gopinathan, 2008). Furthermore, Orensanz *et al*. (1991) recommended that decreased survival rate at high densities was possibly due to food and oxygen depletion, predation or other environmental stresses.

Lower stocking densities can shorten the larval swimming period and reduce the risk of mortality. Therefore, rearing larvae with stocking density at 2,000 individuals/L has been recommended; however, in this study, the food limiting factor might be the cause for low results in growth performance, metamorphose and survival rate of clam larvae. It is necessary to conduct more studies on how to increase feeding parallel together rearing densities to optimize the rearing efficiency in the hatchery.

5 CONCLUSIONS AND RECOMMENDATION

Using 100% fresh algae (*Nannochloropsis sp* and *Chaetoceros sp*) as a food source for mud clam larvae increased shell length, shell width, survival rate, metamorphosis rate, and larvae production.

Rearing mud clam larvae at the density of 2,000 individuals/L showed best results in shell length, shell width increase, survival rate, metamorphose rate, and larvae production.

Further study is needed to increase rearing densities in the range of 2,000 to 8,000 larvae/L with suitable

amount and schedule of feeding to optimize the rearing efficiency in clam hatchery.

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Comparative analysis of the bioactive compound, pigment content and antioxidant activity in different parts of *Pouzolzia zeylanica* plant

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ABSTRACT

Plants are a rich source of therapeutically active compounds such as antioxidants, antibiotics, pigments, vitamins, organic acids, glycosides, and other substances of particular importance to human life. The present study was to analyze and compare the content of bioactive compounds (anthocyanin, flavonoid, polyphenol and tannin); pigments (chlorophyll a, chlorophyll b, total chlorophyll and carotenoids); and antioxidant activity in different parts of Pouzolzia zeylanica plant. The antioxidant activities were evaluated using three methods such as antioxidant ability index, ferrous reducing ability power, and scavenging capacity 2,2-diphenyl-1-picrylhydrazyl radical. The results showed that the content of anthocyanin, flavonoid, polyphenol and tannin of young shoots was significantly ($P<0.01$) higher than that of other parts. In constrast, the content of pigments such as chlorophyll a, chlorophyll b, total chlorophyll and carotenoids of leaves was higher than that of young shoots, whole plants and stems. Besides, the antioxidant capacity of young shoots was also higher than that of leaves, whole plants and stems when performed with three assay methods. It was a correlation between the content of bioactive compounds and antioxidant activities of different parts of Pouzolzia zeylanica plant.

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1 INTRODUCTION

Plants possess various antioxidants which play an important role in the prevention of diseases. It is widely used in many indigenous systems of medicine for therapeutic purposes and increasingly becomes popular in modern society as alternatives to synthetic medicines. Medicinal plant is generally cheaper, accessible or available and are accepted by many people because of the belief that they cause less side effects than some synthetic drugs (Carlson, 2002; Dey and De, 2015).

Pouzolzia zeylanica (L.) Benn. is a perennial herbaceous plant belonging to the *Urticaceae* family, and it is distributed in tropical and subtropical regions. Nowadays, it is present in many Asian countries such as China, India, Indonesia, Japan, Malaysia, Myanmar, Pakistan, Philippines, Sri Lanka, Thailand, Vietnam, Singapore, and some other places in the world (Adhikari and Babu, 2008). It has long been used as one of the components in herbal remedies for treating various diseases by traditional method such as poultices to cure bone fractures, boils and itching; juices or extracts to treat eyes injuries; dysentery and loose stools of infant,

stomach ailments, diabetes, cancer, preventive radiation and confirmed the therapeutic value of polyphenols contained in the leaves (Li, 2006; Yusuf *et al*., 2006; Purkayastha *et al*., 2007; Bhattacharjya and Borah, 2008; Ratnam and Raju, 2008; Mondal *et al*., 2013; Sandhya *et al*., 2013).

In Vietnam, this plant is popularly cultivated in the Mekong Delta; it can be used as fresh or dried plant, decoction drunk to treat cough up phlegm, pulmonary tuberculosis, sore throat, enteritis, dysentery, diuretic, anti-inflammation, urinary infections, galactopoietic, pulmonary disease, etc. (Vo Van Chi, 2012). In modern medicine, *Pouzolzia zeylanica* is also combined with other herbs that could fight cancer cells, tuberculosis and are good for lungs (Le Thanh Thuy, 2007).

The reported studies not only identified the structure and presence of bioactive compounds but also assessed the antimicrobial, antifungal, antioxidant properties of *Pouzolzia zeylanica* plant. However, the chemical components of this medicinal plant in different parts have not been studied yet. The aim of study was to analyze and compare the content of bioactive compounds (anthocyanin, flavonoid, polyphenol and tannin), pigments (chlorophyll a, chlorophyll b, total chlorophyll and carotenoids), and antioxidant activity (AAI – antioxidant ability index, FRAP – ferrous reducing ability power and DPPH – 2,2-diphenyl-1-picrylhydrazyl) of ethanol extract from different parts (shoot, leaf, stem and whole plant) of *Pouzolzia zeylanica*.

2 MATERIALS AND METHODS

2.1 Equipment and chemicals

Equipment used in the study included a spectrophotometer (SPUVS, model SP-1920, Japan), vortex lab (VELP Scientifica, Europe), centrifugal (model EBA 20 Hettich, Germany) and water bath (Menmert, France).

Chemicals that consisted of folin-cioalteau reagent, folin-denis reagent, gallic acid, quercetin, tannic acid, 2,4,6-tri (2-pyridyl)-s-triazine (TPTZ), DPPH and ferrous sulfate were supplied by Sigma Chemical Co. (St. Louis, Mo. USA) and Merck (Darmatadt, Germany). Ferric chloride, aluminum chloride, sodium carbonate, sodium acetate, glacial acetic acid, hydrochloric acid and ethanol were supplied by Analytical Reagent (Xilong Chemical Co. Ltd., China) and Himedia (Hemidia Laboratories Pvt. Ltd., India).

2.2 Sample preparation and extraction

Whole plants of *Pouzolzia zeylanica* were collected at the stage of three months of age after being planted from the experimental area of An Giang University, during June, 2016. The height of plants was about 30-35 cm. Then, the shoots, stems and leaves of plants were separated into different parts. Young shoots were taken from the shoot moristems with a length of about 5 cm. The remaining plants were divided into the leaves and stems (Figure 1).

Fig. 1: Whole plants of *Pouzolzia zeylanica* (a), stems (b), shoots (c) and leaves (d)

The samples were cut fine, taking about 5 g of each plant part to extract with extraction conditions including the ethanol concentration of 60% (v/v), ratio of material to solvent of 1/20 (g/mL), extraction time of 60 minutes and temperature of 60^{o}C (Nguyen Trong Diep *et al*., 2013; Nguyen Tien Toan and Nguyen Xuan Duy, 2014). The triangular flask with cover and thermostatic tank were used in this research. The extract was filtered using Buchner funnel with Whatman's No 1 filter paper. The filtrate (crude extract) was diluted in ethanol at an appropriate ratio using for analysis.

2.3 Analytical methods

2.3.1 Determination of anthocyanin content

Total monomeric anthocyanin content was determined following different pH method (Lee *et*

al., 2005; Ahmed *et al.,* 2013); the result was expressed in milligrams of cyanidin-3-glucoside equivalents (CE) per gram of dry weight (DW). Sample absorbance was read against a blank cell containing distilled water. The absorbance (A) of the sample was then calculated according to the following formula:

$$A = (A_{520} - A_{700})\ pH_{1.0} - (A_{520} - A_{700})\ pH_{4.5}$$

Where A_{520} and A_{700} are absorbance of sample in the two pH buffer solutions ($pH_{1.0}$ and $pH_{4.5}$) at the wavelenght λ = 520 and 700 nm.

The total anthocyanin content (TAC) in the original sample was calculated according to the following formula:

$$\text{TAC (mg CE/g DW)} = \frac{[A \times MW \times DF \times 1000] \times V}{(\varepsilon \times 1) \times W}$$

Where MW is cyanidin-3-glycoside molecular weight (449.2 in g/mol); DF is the dilution factor; V is volume of the obtained extracts (L); ε is molar absorptivity (26,900 in L/mol); W is the weight of material sample (g).

2.3.2 Determination of flavonoid content

Aluminum chloride colorimetric method was used for flavonoids determination (Eswari *et al*., 2013; Mandal *et al*., 2013). About 1 mL of the crude extracts/standard of different concentration solution was mixed with 3 mL of ethanol, 0.2 mL of 10% aluminum chloride, 0.2 mL of 1 M sodium acetate and 5.8 mL of distilled water. It remained at room temperature for 30 minutes. The absorbance of the reaction mixture was measured at 415 nm with spectrophotometer against blank. The calibration curve was prepared by diluting quercetin in ethanol ($y = 0.0054x + 0.0026$ and $r^2 = 0.9995$). The total flavonoid content (TFC), milligrams of quercetin equivalents (QE) per gram dry weight (DW), was calculated by the following formula:

$$\text{TFC (mg QE/g DW)} = \frac{[A - 0.0026] \times DF \times V}{0.0054 \times W}$$

Where A is the absorbance of the test samples; DF is the dilution factor; V is volume of the obtained extracts (L); W is the weight of material sample (g).

2.3.3 Determination of polyphenol content

Total polyphenol content was determined by folin-ciocalteu reagent method (Hossain *et al*., 2013). Each crude extract (0.2 mL) was taken in a test tube and added 10% Folin-Ciocalteu reagent (1.5 mL). Then all test tubes were kept in a dark place for 5 minutes. Finally, 5% Na_2CO_3 (1.5 mL) was added to solution and mixed well in a vortex. Again, all the test tubes were kept in the dark for 2 hours. The absorbance was measured for all solution by using UV-spectrophotometer at constant wavelength of 750 nm. Total polyphenol concentrations were quantified by calibration curve obtained from measuring the absorbance of a known concentration of gallic acid standard in ethanol ($y = 0.0082x + 0.0595$ and $r^2 = 0.9996$). The total polyphenol content (TPC), milligrams of gallic acid equivalents (GAE) per gram dry weight (DW), was calculated by the following formula:

$$\text{TPC (mg GAE/g DW)} = \frac{[A - 0.0595] \times DF \times V}{0.0082 \times W}$$

Where A is the absorbance of the test samples; DF is the dilution factor; V is volume of the obtained extracts (L); W is the weight of material sample (g).

2.3.4 Determination of tannin content

Tannin content was determined by folin-denis method (Laitonjam *et al.,* 2013). Each crude extract (0.5 mL) and distilled water (0.5 mL) were taken in a test tube. Finally, the samples were treated with 0.5 mL of freshly prepared folin-denis reagent, and 20% sodium carbonate (2 mL) was added, shaken well, warmed on boiling water-bath for 1 minutes and cooled to room temperature. Absorbance of the colored complex was measured at 700 nm. Tannin concentration was quantified basing on the calibration curve of tannic acid in ethanol ($y = 0.0098x + 0.0478$ and $r^2 = 0.9996$). The tannin content (TC), milligrams of tannic acid equivalents (TAE) per gram dry weight (DW), was calculated by the following formula:

$$\text{TC (mg TAE/g DW)} = \frac{[A - 0.0478] \times DF \times V}{0.0098 \times W}$$

Where A is the absorbance of the test samples; DF is the dilution factor; V is volume of the obtained extracts (L); W is the weight of material sample (g).

2.3.5 Determination of AAI

AAI of samples were determined by reducing power method (Nguyen Thi Minh Tu, 2009; Saha *et al*., 2013). Two ml of plant extract was mixed with 2.5 ml phosphate buffer (pH 7.4) and 2.5 ml of 1% aqueous postassium ferriccyanide solution. This mixture was kept at 50°C in water bath for 20 minutes. After cooling, 2.5 ml of 10% trichloroacetic acid was added and centifuged at 3,000 rpm for 5 minutes. The supernatant (2.5 ml) was mixed with distilled water (2.5 ml) and 0.5 ml of 0.1% freshly prepared ferric chloric solution. Then the absorbance of solution was measured at 700 nm using a spectrophotometer against blank. AAI calculated by the following formula:

AAI = Abs sample/Abs blank

Where Abs sample is the absorbance of extract; Abs blank is the absorbance of distilled water

2.3.6 Determination of FRAP

FRAP assessment was performed according to the method of Adedapo *et al.* (2009). The stock solutions included 300 mM acetate buffer (pH 3.6), 10 mM TPTZ (2, 4, 6-tripyridyl-*s*-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ solution. The fresh working solution was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ, and 2.5 ml $FeCl_3 \cdot 6H_2O$. The temperature of the solution was raised to 37°C before use. Plant extracts (150 µL) were allowed to react with 2,850 µl of the FRAP solution
for 30 minutes in the dark condition. Readings of the colored product (ferrous tripyridyltriazine complex) were taken at 593 nm. The standard curve of $FeSO_4$ was established (y = 0.5177x + 0.0855 and r^2 = 0.9981). Results were expressed in µM $FeSO_4$/g dry weight (DW).

$$\text{FRAP (µM } FeSO_4\text{/g DW)} = \frac{[\text{Abs} - 0.0855] \text{ x V x } 1000}{0.5177 \text{ x W}}$$

Where Abs is the absorbance of sample; V is volume of the obtained extracts (L); W is the weight of material sample (g).

2.3.7 Determination of DPPH radical scavenging capacity

The scavenging ability of extract against DPPH radical was determinaed using the method of Aluko *et al.* (2014). One millilitre of 0.135 mM of DPPH in ethanol was mixed with 1 ml of test solution. The mixture was kept in a dark cupboard for 30 minutes. The absorbance of the resulting solution was measured spectrophotometerically at 517 nm and the scavenging ability of the extract was calculated as:

DPPH radical scavenging activity (%) = [(Abs control – Abs sample)/Abs control] x 100

Where Abs control is the absorbance of DPPH radicals + ethanol; Abs sample is the absorbance of DPPH radical + extract

2.3.8 Determination of pigments content

The content of chlorophyll and carotenoids of samples were performed according to the method of Singh *et al.* (2014). Sample extracts were measured at 663, 645 and 480 nm wavelengths, with 60% ethanol as the blank. The chlorophyll content was calculated by the following formula:

Chlorophyll a (mg/g DW) = [(12.7x A_{663} – 2.69 x A_{645})/(1000 x W)] x V

Chlorophyll b (mg/g DW) = [(22.9 x A_{645} – 4.68 x A_{663})/(1000 x W)] x V

Total chlorophyll (mg/g DW) = [(20.2 x A_{645} – 8.02 x A_{663})/(1000 x W)] x V

Carotenoids (mg/g DW) = A_{480} + (0.114 x A_{663}) – (0.638 x A_{645})

Where A is the absorbance of the extract at respective wavelengths, V is the volume of extract (ml), and W is the weight of the sample (g)

2.4 Data analysis

All results were presented as means and standard deviation. A statistical analysis system (Statgraphic software package, version 16.0) was used to perform all statistical analyses. Data were compared by one-way analysis of variance; the analysis of LSD was considered significantly different at P≤0.05.

3 RESULTS AND DISCUSSION

Almost all of the parts of the plants namely leaf, flower, fruit, stem and root have their own bioactive compounds which can be used for therapeutic purpose. Typically, medicinal plants ensure an extensive supply of antibiotic, antifungal, antiseptic, analgesic compounds etc. (Pandurangan *et al.,* 2018). Several studies reported that the aerial parts of the plants, such as stems and leaves, are normally used for the extraction of active phytochemicals. According to previous findings of medicinal herbs researches, there are some determining factors of the amount and types of phytochemicals content. Other researchers claimed that growth stage of plants contributes to the level of phytochemical content (Raya *et al.,* 2015). *Pouzolzia zeylanica* has been known as medicinal plant which contains various bioactive comppounds such as polyphenol, flavonoid, tannin, isoflavone, glycoside, phyllanthin, vitexin, carotenoids, etc. (Ghani, 2003; Le Thanh Thuy, 2007; Saha and Paul, 2012). The result of the present study showed that the content of bioactive compounds in different parts of *Pouzolzia zeylanica* plant was different (Table 1).

Table 1: The content of bioactive compounds in different parts of *Pouzolzia zeylanica*

Different parts	Anthocyanin (mgCE/g DW)	Flavonoid (mgQE/g DW)	Polyphenol (mgGAE/g DW)	Tannin (mgTAE/g DW)
Young shoots	3.12 ± 0.132^{a}	18.72 ± 0.487^{a}	39.32 ± 1.526^{a}	29.54 ± 0.568^{a}
Leaves	2.65 ± 0.059^{b}	17.39 ± 0.165^{b}	32.47 ± 0.926^{b}	26.87 ± 0.508^{b}
Stems	0.89 ± 0.039^{d}	6.68 ± 0.497^{d}	20.06 ± 0.975^{c}	20.75 ± 0.941^{c}
Whole plants	2.06 ± 0.082^{c}	14.88 ± 0.166^{c}	30.53 ± 1.031^{b}	26.18 ± 0.722^{b}

Note: Data represent the means (n=3) and ± standard deviation. Values in each column followed by the same super-script letters are not significantly different by LSD at P≤0.05.

Phenolic compounds are secondary metabolites and naturally present in plants. They have great importance for the food and drink products derived from plants, since these compounds are responsible for their organoleptic properties (Dvořáková *et al*., 2007). Anthocyanins are responsible for attractive colors of flowers, fruits and vegetables as well as their products (Mazza and Brouillard, 1990). In addition, anthocyanin also have multiple biological roles, e.g. antioxidant activity, anti-inflammatory action, inhibition of blood platelet aggregation and antimicrobial activity, treatment of diabetic retinopathy and prevention of cholesterol-induced atherosclerosis (Mazza and Miniati, 1993; Wang *et al*., 1997; Cliford, 2000; Espin *et al.,* 2000). Flavonoids can have a wide range of biological activities, the protective role of flavonoids in living systems was mostly due to their antioxidant potential, which is related to transfer of reactive oxygen species, chelation of metal catalysts, activation of antioxidants enzymes and inhibition of certain type of oxidases and colon cancer (Heim *et al*., 2002; Chidambara Murthy *et al*., 2012). Flavonoids also have the potency to stimulate the immune system, induce protective enzymes in the liver or block damage to genetics materials (Zarina and Tan, 2013). Polypenols are present in various plants and have been shown to be good antioxidant in both in vitro and in vivo studies. It helps reduce the risk for various life style-related diseases including cancer and cardiovascular diseases, which have been linked to the formation of active oxygen species (Yoshida *et al.,* 2000). Tannin is present in varying concentrations in plants, and plays important roles in modulating cardiac action potential repolarization and tumor cell biology (Chu *et al*., 2015).

The results in Table 1 showed that the content of anthocyanin and flavonoid in whole *Pouzolzia zeylanica* plant was 2.06±0.082 mg CE/g DW and 14.88±0.166 mg QE/g DW, respectively, and there was statistically significant difference between parts of plants such as young shoots, leaves, stems and whole plants with P≤0.01. In particular, young shoots contained the highest anthocyanin and flavonoid content, with 3.12±0.132 mg CE/g DW and 18.72±0.487 mg QE/g DW, followed by leaves, whole plants and stems. Similarly, the highest content of polyphenol and tannin were recorded in young shoots, with 39.32±1.526 mg GAE/g DW and 29.54±0.568 mg TAE/g DW, followed by leaves and whole plants, and there was no statistically significant difference between leaves and whole plants (P≤0.01). The lowest content of these compounds was observed in stems. The result of Raya *et al.* (2015)'s study also showed that the content of total phenolic and flavonoid in *Clinacanthus nutans* were significantly influenced by plant parts. The content of these compounds was higher in leaves than that in stems. Quantification of secondary metabolites in the root, stem and foliar tissues of *Centella asiatica* revealed the presence of various bioactive compounds at varying concentrations. The concentrations of phenols, tannin and flavonoid was higher in the leaves than that in stems and roots (Vaddadi *et al.,* 2017). The phenolics content of *Moringa oleifera* plant was higher in leaf than that in stems and stalks (Shih *et al.,* 2011). Each plant part has different content of chemical substances, for example, total phenolic content and antioxidant composition of *Urtica dioica* L. vary with plant parts (Khare *et al.*, 2012).

Phenolic compounds of the extracts are probably involved in their antiradical activity. Phenolic compounds have an important role in stabilizing lipid oxidation and are associated with antioxidant activity because of their scavenging ability due to their hydroxyl groups (Shih *et al.,* 2011). A rapid, simple and inexpensive method to measure antioxidant capacity of food involves the use of the free radical, DPPH. DPPH is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors, and to evaluate antioxidant activity of plant extracts. One important mechanism of antioxidation involves the scavenging of hydrogen radicals. DPPH has a hydrogen free radical and shows a characteristic absorption at 517 nm. After encountering the proton-radical scavengers, the purple color of the DPPH solution fades rapidly (Deighton *et al*., 2000). The method of AAI assay showed that antioxidants can donate an electron to free radicals,

which leads to the neutralization of the radical. Reducing power was measured by direct electron donation in the reduction of $Fe^{3+}(CN-)_6$–$Fe^{2+}(CN-)_6$. The extract was visualized by forming the intense Prussian blue color complex and then measured at λ 700 nm (Yen and Chen, 1995). In addition, FRAP assay measures the reducing potential of an antioxidant reacting with a ferric tripyridyltriazine [Fe^{3+}-TPTZ] complex and producing a coloured ferrous tripyridyltriazine [Fe^{2+}-TPTZ] (Benzie and Strain, 1996). Generally, the reducing properties are associated with the presence of compounds which exert their action by breaking the free radical chain by donating a hydrogen atom (Duh *et al.,* 1999). FRAP assay treats the antioxidants in the sample as a reductant in a redox-linked colorimetric reaction (Guo *et al.,* 2003). The ethanol extracts of different parts of *Pouzolzia zeylanica* plant were able to reduce the unstable radical DPPH to the yellow-colored diphenylpicrylhydrazine. The results of the evaluation of the antioxidant activity of various plant parts were presented in Table 2.

Table 2: Antioxidant activity and moisture in different parts of *Pouzolzia zeylanica*

Different parts	AAI	DPPH (%)	FRAP (µM FeSO4/g DW)	Moisture (%)
Young shoots	5.52 ± 0.172^a	88.29 ± 0.942^a	578.10 ± 8.371^a	83.23 ± 0.589^c
Leaves	4.84 ± 0.077^b	85.14 ± 1.184^b	529.08 ± 10.101^b	82.67 ± 0.406^c
Stems	3.93 ± 0.111^c	58.56 ± 0.799^d	501.20 ± 6.843^c	86.97 ± 0.155^a
Whole plants	4.71 ± 0.060^b	78.11 ± 1.264^c	546.11 ± 5.171^b	85.28 ± 0.094^b

Note: Data represent the means (n=3) and ± standard deviation. Values in each column followed by the same superscript letters are not significantly different by LSD at P≤0.05.

Table 2 showed that ethanol extract of young shoots had the highest antioxidant activity among the three tested methods, followed by leaves, whole plants and stems (AAI method), and followed by whole plants, leaves and stems (FRAP method), and there was no statistically significant difference between leaves and whole plants. While there was statistically significant difference (P≤0.01) in various parts such as young shoots > leaves > whole plants > stems (DPPH method). The lowest antioxidant value was found in stems. For example, the young shoots extract had AAI of 5.52; scavenging 88.29% free radical of DPPH and 578.10 µM $FeSO_4$/g DW. The study result of Raya *et al.* (2015) showed that antioxidant power was higher in young plant than that in old plant irrespective of plant parts. The highest DPPH was observed in young leaves followed by young stems. The lowest DPPH was recorded with matured stems. Ethanol extracts of *Centella asiatica* root, stem and leaf were tested for their scavenging activities. Result showed that leaf extracts have shown high DPPH scavenging activities compared with those of root and stem extracts (Vaddadi *et al.,* 2017). The methanolic extract of Moringa showed strong scavenging effect of DPPH radicals and reducing power. The trend of antioxidative activity as a function of the part of *Moringa oleiferwas*: leaf > stem > stalk for samples investigated (Shih *et al*., 2011).

The analysis of the moisture content of different parts of *Pouzolzia zeylanica* plant showed that the highest moisture content was observed in stems, followed by whole plants, young shoots and leaves. There was statistically significant difference (P≤0.01) between these parts of plant. The moisrure content ranged from 82.67 to 86.97% (Table 2).

Chlorophyll is a specifically pigment of green plants, which plays a key role in photosynthesis. In plants there are several types of chlorophyll, denoted by letters of a, b, c, d. Chlorophyll has effects on the human body. External acts as deodorant and skin tonic, internally, stimulates respiration, helps in cleansing waste and helps combat anemia (Dumbrava *et al*., 2012). The major cholorophylls in plants include choloropyll a and cholorophyll b, which are usually present at a ratio of 3 (Chen and Chen, 1993). Chlorophyll a is recognized as the main pigments which convert light energy into chemical energy. Chlorophyll b as accessory pigments acts indirectly in photosynthesis by transferring the light that it absorbs to chlorophyll a. The chlorophyll molecule has Mg^{2+} at its center which makes it ionic and hydrophilic, and a ring that is hydrophobic in nature with a carbonyl group at its tail which makes it polar. It is held in place in the plant cell within a water-soluble chlorophyll-binding protein. Chlorophyll-b differs from chlorophyll-a only in one functional group (i.e -CHO) bounded to the porphyrin ring, and is more soluble than chlorophyll-a in polar solvents because of its carbonyl group (Costache *et al.,* 2012; Sumanta *et al.,* 2014). Carotenoids are located in chromoplast, contribution color to vegetables/fruits, and also in chlorophylls, where together with chlorophylls involved in the two photosystems. Carotenoids group and their derivatives consist of about 70 compounds that are present in most vegetables and fruits. The carotene pigments were the most important photosynthetic

pigments, and they prevented chlorophyll and thylakoid membrane from the damage of absorbed energy by peroxidation (Costache *et al.,* 2012; Sumanta *et al.,* 2014). Analytical result in this study showed that *Pouzolzia zeylanica* plant was also present chlorophylls and carotenoids pigments (Table 3).

Table 3: The content of pigments in different parts of *Pouzolzia zeylanica*

Different parts	Chlorophyll a (mg/g DW)	Chlorophyll b (mg/g DW)	Total chlorophyll (mg/g DW)	Carotenoids (mg/g DW)
Young shoots	2.203 ± 0.073^{a}	1.601 ± 0.066^{b}	3.802 ± 0.138^{b}	7.725 ± 0.096^{b}
Leaves	2.292 ± 0.068^{a}	2.164 ± 0.104^{a}	4.455 ± 0.038^{a}	8.152 ± 0.020^{a}
Stems	0.681 ± 0.015^{c}	0.690 ± 0.029^{d}	1.371 ± 0.043^{d}	3.171 ± 0.089^{d}
Whole plants	1.375 ± 0.062^{b}	1.056 ± 0.048^{c}	2.430 ± 0.110^{c}	5.128 ± 0.167^{c}

Note: Data represent the means (n=3) and ± standard deviation. Values in each column followed by the same superscript letters are not significantly different by LSD at P≤0.05.

Table 3 showed that the highest content of chlorophyll a was observed in leaves, with 2.292±0.068 mg/g DW, followed by young shoots, whole plants and stems, and there was statistically significant difference between leaves, whole plants and stems, but there was no statistically significant difference between leaves and young shoots. The highest content chlorophyll b, total chlorophyll and carotenoids were also recorded in leaves, with 2.164±0.104 mg/g DW, 4.455±0.038 mg/g DW, 8.152±0.020 mg/g DW, respectively, followed by young shoots, whole plants and stems, there was statistically significant difference between these different parts (P≤0.01). In the tested samples a ratio between chlorophyll a and chlorophyll ranged from 0.99 to 1.38, meaning that chlorophyll a was the main form of chlorophyll in young shoots, and chlorophyll b was the main form of chlorophyll in stems. Other scientists also reported that changes in the color and the content of chlorophylls were related to the genotype but not to the growing conditions (Bekhradi *et al*., 2015). The result of the present study was in line with the reported result of Straumite *et al.* (2015), in the stems chlorophyll content was significantly lower than in leaves. The highest chlorophyll content was observed in young leaves which contained 72% higher chlorophyll than matured leaves. The lowest chlorophyll content was found in matured stems (Raya *et al.*, 2015). The basic pigments of green plants are chlorophylls, always accompanied by carotenoids. In part of samples, significantly higher concentration of carotenoids in stems was observed (*Mentha suaveolens*) and significantly higher content of carotenoids in leaves only in *Mentha piperita* was determined. For other samples, differences between the leaves and the stems were not significant (Straumite *et al.,* 2015).

4 CONCLUSIONS

The content of bioactive compounds, pigments and the antioxidant activity of *Pouzolzia zeylanica* plant were differently present in various parts of plant. The quality characteristics of young shoots were higher than those of leaves, whole plants and stems. The content of anthocyanin, flavonoid, polyphenol, tannin, chlorophyll a, chlorophyll b, total chlorophyll and carotenoids in young shoots was 3.12 mg CE/g DW, 18.72 mg QE/g DW, 39.32 mg GAE/g DW, 29.54 mg TAE/g DW, 2.203 mg/g DW, 1.601 mg/g DW, 3.802 mg/g DW, 7.725 mg/g DW, respectively. This result showed that young shoots of *Pouzolzia zeylanica* plants can be used to process tea. It can be considered as good sources of natural products that may be employed in the treatment of the different diseases associated to the oxidative stress.

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A simple spectrophotometric method for quantifying total lipids in plants and animals

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ABSTRACT

Lipids play important roles for the cell in storing energy, structuring cell membrane, and signaling pathway. Consequently, lipidis are analyzed routinely in various research fields. In the current research, a reliable, rapid and economical assay has been developed to quantify the total of lipid in various samples. The development of colorimetric sulfo-phospho-vanillin is for high throughput analysis of total lipids. In this method, a reaction mixture is performed in a 96-well microplate. The advantages provided from this new assay over other lipid measurement methods, included only small amount of sample requirement for fitting in the standard range (less than 100 µg/mL), less time requirement and labor in analyzing a large number of sample (about 1 hour), and the more consistent of color development between lipid content and reagent concentration.

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1 INTRODUCTION

Lipids are considered as an important group of compounds providing several biological functions as energy storage, cell membrane structure and signaling (Wymann and Schneiter, 2008). This is the reason why the analysis of lipids is performed routinely in various research areas. For instance, in both research and industry settings, the screening of oleaginous living beings has broad application to recognize and produce food supplements and renewal biofuels (Ratledge, 2002; Ratledge and Cohen, 2008). There are several methods developed for quantifying total lipids. Among them, a macro-gravimetric is the basic technique in which lipids are separated from a sample, the extraction solvent is evaporated, and the remaining extract is estimated as the lipid content (Folch *et al*., 1957; Bligh and Dyer, 1959). It is necessary to have a relatively large quantity of sample in this traditional gravimetrical method. Time-consumption and labor-intension are also required in case the analysis of many samples is needed. Lipid spectrofluorometric analysis using Nile red fluorescent dye is the method that was originally developed by Greenspan *et al*. (Greenspan *et al*., 1985) and has also been modified to quantify the total of lipids (Fowler and Greenspan, 1985; Huang *et al*., 2009). This approach is high-throughput while the environmental factors and other components in the cell cytoplasm, including proteins and pigments, may affect the fluorescence intensity (Chabrol and Charonnet, 1937; Desvilettes *et al*., 1997). Because of this reason, the lipid quantification accuracy by

applying this approach requires a determination of the optimal spectra and reaction conditions for each type of specimen prior to fluorescent measurements (Johnson, *et al*., 1977).

Because of its fast response and relative ease in sample handling, the colorimetric sulfo-phospho-vanillin (SPV) method developed by Chabrol and Charonnet (1937) is considered as an attractive alternative for lipid measurement (Chabrol and Charonnet, 1937; Johnson *et al*., 1977). The adjustment of SPV method has been executed for various applications, for example, the examination of total lipids in serum, sustenance and biological examples (Nakamatsu and Tanaka, 2004; Haskins *et al*., 2010). A micro-scale modification of the SPV assay was developed by Van Handel (1985) to determine the total lipids in a single mosquito, and assessed by several investigators as an efficient approach in time and labor compared to the gravimetric method (Lasorsa and Casas, 1996; Lu *et al*., 2008). Because of the continuous development of color, it is important to handle the sample carefully and control the color development in using this micro-scale approach. From the results of previous works, it is necessary to have an adaptation of the SPV method in order to complete the lipid quantification in a 96-well microplate for higher throughput and reduced costs. This adapted method requires an assay in which the reagent mixture is confined to one microplate for the entire assay. This prompts the faster estimation of different examples with easy background correction and the more reliable checking of color development. For example, the application of this assay method on soybean oil and triolein as a standard has successfully measured the total lipids in extracts from fruit flies, which contain lipid in the 3rd instar larvae. The objective of this study is to establish the method for quantifying total lipid from samples.

2 MATERIALS AND METHODS

2.1 Chemicals

All chemicals used were from Japan. Soybean oil and triolein are standard lipids as well as methanol and sulfuric acid were from Sigma; others at analytical grade including sodium sulfate (Na_2SO_4), chloroform, vanillin, and phosphoric acid were from Wako.

2.2 Preparation of phospho-vanillin reagent

Vanillin (0.6 gram) was dissolved in 100 mL of hot distilled water (vanillin reagent), then vanillin reagent was mixed with 400 mL phosphoric acid (85%). The phospho-vanillin reagent was stored in a brown bottle at room temperature (Kaufmann and Brown, 2008).

2.3 Preparation of lipid standard

Soybean oil (vegetable oil) and triolein were used as the standards for the colorimetric method in surveying the applicability of the assay. The content of total lipid in plants usually has a higher unsaturated proportion and is relatively close in composition to vegetable oil, concurrently triolein is a symmetrical triglyceride which is structured from three units of the unsaturated fatty acid oleic acid and glycerol. Most triglycerides are unsymmetrical and derived from mixtures of fatty acids. The main constituent of vegetable oil and animal fats is triglycerides. In this work, soybean oil was tested in the range of 0 – 100 µg/mL, and triolein was tested in the range of 0 – 125 µg/mL. There are two of these standards which were dissolved in chloroform and added in 96-well microplate. After the evaporation of chloroform at 90°C for 20 minutes, 50 µL concentrated sulfuric acid (98%) was added to each well, and then the microplate was incubated at 90°C for 20 minutes. A volume of 150 µL vanillin–phosphoric acid reagent was added to each well for color development. After 10 minutes, the absorbance at 530 nm was measured using a SH-1200 microplate reader (Corona Electric, Japan). This method is based on the reaction of lipids with concentrated sulfuric acid at high temperature to form carbonium ions, then these ions subsequently react with the vanillin phosphate ester to yield a pink-colored complex which is examined photometrically (Frings and Dunn, 1970). The ion formed is stable on cooling down at room temperature for at least several hours. The condition for a positive SPV reaction requires the presence of double bonds or free hydroxyl groups within the lipid analytes (Johnson *et al*., 1977).

2.4 Extraction of total lipid in animal tissue

The 3rd instar larvae *Drosophila melanogaster* (from 1 to 5 flies) was homogenized in 100 µL of 2% sodium sulfate, and then 900 µL of chloroform/methanol (1:1) was added. The supernatant was collected by centrifugation (10,000 rpm, 5 minutes), mixed with 300 µL of distilled water, and centrifuged again (10,000 rpm, 5 minutes). For lipid measurement, the chloroform layer was transferred into 96-well microplate and dried at 90°C to evaporate the chloroform (about 20 minutes), and then 50 µL of 98% sulfuric acid was added, and the solution was incubated for 20 minutes at 90°C. A volume of 150 µL vanillin–phosphoric acid reagent was added to each well for color development. After cooling down to room

temperature for 10 minutes, absorbance was measured at 530 nm.

2.5 Data analysis

Linearity was determined by plotting absorbance versus lipid amount in the assay and examining the R^2 value upon linear regression of the data. The error bars represent the standard deviation.

3 RESULT AND DISCUSSION

3.1 Relative absorbance and linearity of standard lipid samples

Depending on previous report by Ahlgren and Merino (1991), the selection of an appropriate standard was important to assess lipid content in different types of samples. Therefore, soybean oil and triolein are two types of standards which were tested using a new assay format (Fig. 1 and Fig. 2). This assay system shows that soybean oil has driven an increase in absorbance. The result from Fig.1 showed that this method is sensitive and adaptable to measure a small amount of lipid in the sample. The concentration of soybean oil used is from 0-100 µg/mL, and the absorbance dramatically increased in the well having a high concentration of soybean oil. In this assay, soybean oil was used as a standard for lipid from plants. This method is suitable to measure the total of lipids from plant samples according to the result.

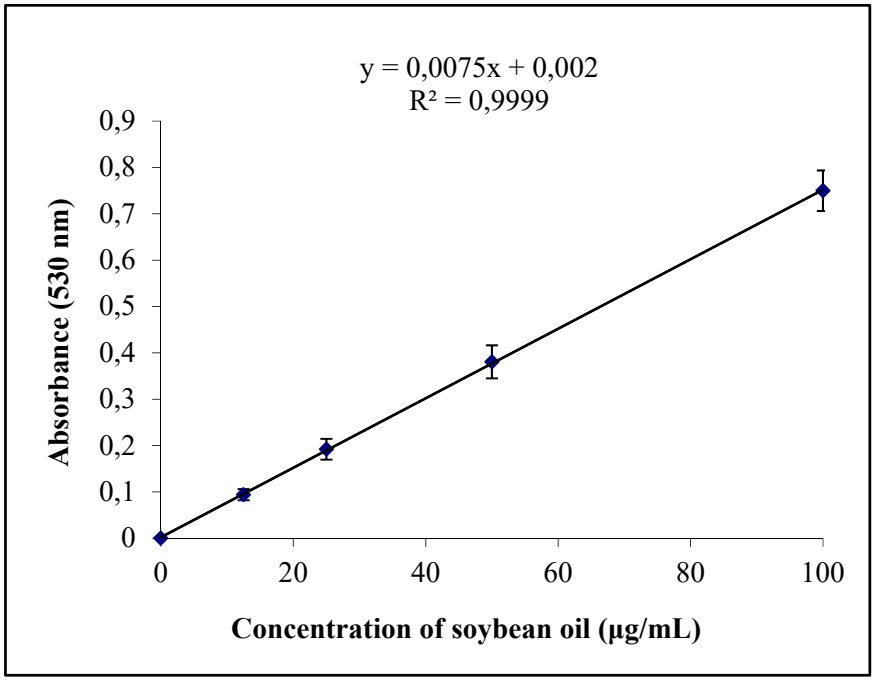

Fig. 1: Lipid measurement by the coupled colorimetric test

The linear relationship between the absorbance and soybean oil concentration (0 - 100 µg/mL). Soybean oil measured by the SPV method with a correlation coefficient of 0.9999 and regression equation of y = 0.0075x + 0.002. Each point in the regression represents the replicate measurement (n = 3).

In the current study, triolein was used as a standard. It is an unsaturated lipid which reacts with a good yield. The method easily manipulated and inexpensive reagents can be purchased in many chemical companies (Izard and Limberger, 2003). Fig.2 shows the correlation of lipid concentration (triolein) and absorbance using the SPV method. Depending on the spectrophotometric result in a linear increase (R^2 = 0.9924) of absorbance values, it indicates that the assay allows a reliable lipid measurement in this concentration range.

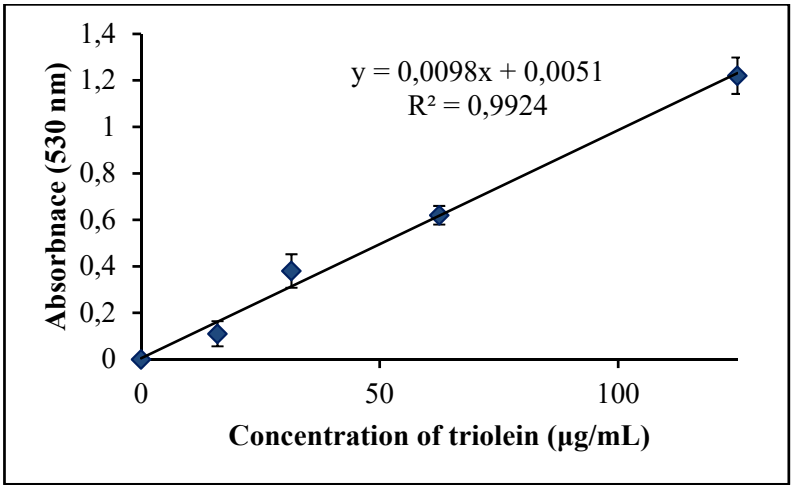

Fig. 2: Reliable lipid determination by the coupled colorimetric assay

Linear absorbance increase of triolein (0 - 125 µg/mL) and measured by SPV method with a correlation coefficient of 0.9924 and regression equation of y = 0.0098x + 0.0051. Data points and error bars represent the mean and standard deviation of four replicate samples.

3.2 Total lipid in animal sample

DNA, RNA, and proteins did not detectably react or interfere with the SPV, the reagents used for lipid extraction as described in this report also did not affect the assay (Izard and Limberger, 2003). The SPV reaction detected microgram level of lipids (as shown in Fig. 1 and Fig. 2). The wavelength was referenced to measure the absorbance of the sample is 530 nm. It was selected based on the wavelength of maximal absorption on tested lipid standards and total lipid extracted from fruit flies (data not shown), and this wavelength was also used for the determination of lipid in some previous reports (Folch *et al*., 1957; Izard and Limberger, 2003). In this assay, chloroform/methanol was used to extract total lipid from the 3rd instar larvae of *Drosophila melanogaster*. Lipids exist in form of unsaturated compounds (*or move to introduction of lipids*) that do not dissolve in polar solvents like water but are highly soluble in the non-polar or weakly polar organic solvents, including chloroform, ether, benzene, and acetone (Reis *et al*., 2013). Fig. 3 shows that there is a relationship between the lipid extracted from fruit flies and the absorbance at 530 nm (R^2 = 0.9924). This result explained that the SPV method is suitable for measuring total lipid from animal samples.

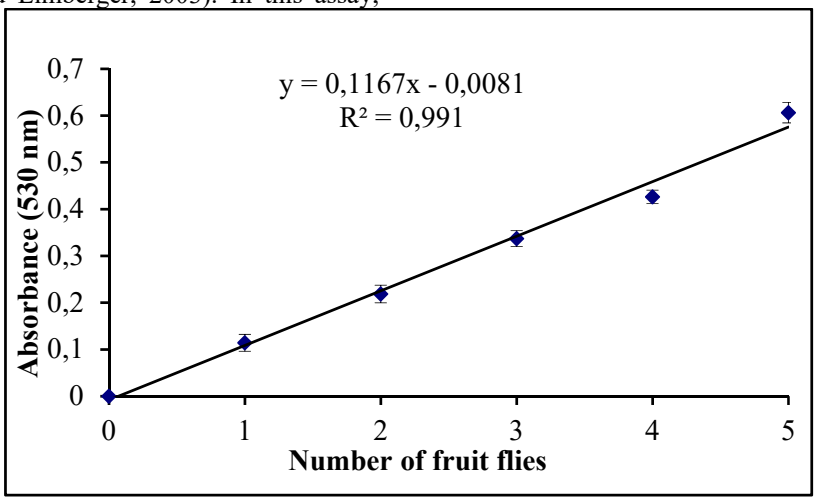

Fig. 3: SPV measurement accuracy depends on the number of flies per assay

Data showed total lipid measurements of three replicates each of group from 1 to 5 fruit flies (yellow white flies). Absorbance measurements were made after 10 minutes of color development. Data points represent the mean of three replicate samples.

In various studies, the total lipid quantification was frequently performed with different kinds of samples. The present work reports a modified colorimetric method for quantitative analysis of total lipid using a high throughput microplate format, where extracted and purified lipid from samples was used. The extraction procedure limited the interferences associated with other components in the sample and allowed different samples from various research areas to be analyzed in the same conditions.

4 CONCLUSIONS

There are advantages of the new assay method, including (1) it just uses a small amount sample and the sample volume can be adjusted to fit in the standard range, (2) it requires less time (<2 hours) and less labor when a large number of samples is analyzed, and (3) the color development is more consistent between lipid contents and reagent concentrations. Moreover, the reagents used in this assay are inexpensive and easy for the handle. In the final procedure it is recommended that the volume of the sample should be less than 100 µL in order to ensure a complete reaction with the sulfuric acid. In addition, uniform heating and cooling are important for consistency in the reaction. When soybean oil and triolein were used as standards, this method can be used to measure the total lipid in different samples including cell cultures, plants, and animal tissues.

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Transformative learning in resilient VACB model adapting to climate change in Phong Dien district, Can Tho city

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Climate change, livelihood, resilient, transformative learning, VACB

ABSTRACT

This study has assessed transformative learning in the VACB (V is orchard, A is pond, C is livestock and B is biogas) livelihood model of Phong Dien district, Can Tho city to propose solutions for maintaining and promoting transformative learning sustainably. Mxed method was used including surveys, interviews, focus group discussions and expert observation and discussion as main data collection. The study has obtained some following results. Firstly, the local livelihood in Phong Dien has changed dramatically. Secondly, there were seven different types in transformative learning in this area consisting of self-learning, and learning through workshop, training, model-observation, community activities, media tools and picking up. Thirdly, transformative learning process in Phong Dien faced a number of difficulties related to residents' aptitude and awareness, local government support, lack of information and learning space. Based on above difficulties and practical observation on the research issues, a number of solutions were proposed to promote transformative learning in Phong Dien including promoting representative farmers' roles, taking advantages of meeting, observing the information from television, internet and social network.

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1 TRANSFORMATIVE LEARNING, LIVELIHOOD AND VACB MODEL

1.1 Transformative learning

Transformative learning is a process of changing social perspectives and actions towards sustainable understanding, beliefs, and lifestyle. According to Mezirow (1997), in the transformative learning process people firstly change their understanding and perceptions, then they intend to change their actions in relation to their living environment. To change human consciousness and actions, it takes time as people have to obtain knowledge and experience thanks to communicating and cooperating with others (Mezirow and Talor, 2009). Thanks to learning themselves and with other people, the human beings usually deal with their works more effectively. So it can be said that transformative learning needs to rely on both individual and social experience, in which people can learn and improve their lifestyle from not only success but also failure. The most important thing is

that transformative learning only takes place in case people communicate, learn and share with their peers or community in particular environment. Moreover, the transformative learning environment needs to support people to think, choose and make their decision (Taylor and Cranton, 2012). Besides, there are some essential conditions that influence transformative learning consisting of social, cultural and economic perspectives, individual and community understanding and information technology. Among them, transformative learning environment plays a vital role in three different criteria. Firstly, it needs to be a large geographic space. Secondly, learning space should consist of some different and complicated fields. Finally, this has to be a learning space for systematic thinking.

Three main components of transformative learning are transformative teachers, transformative learners, and stakeholders. Each of those components has its own importance that can affect transformative learning results. More specifically, transformative teachers play a main role in providing the community with new knowledge. Transformative learners are those who would acquire their new understanding. There are some transformative learners who are excellent in their learning process can play an essential role in supporting other learners. It is noted that in this learning process, the learners have to be active and in the center of their learning tasks to participate and share their ideas in most learning activities. Stakeholders are also necessary as they can support and encourage transformative learners to join in community learning tasks (Mezirow, 1997; Taylor and Cranton, 2012). Additionally, national and international companies and organizations, universities, vocational education schools and institutes should help both transformative teachers and transformative learners to carry out their roles.

Transformative learning is a process that people can experience their real-life situations consisting of individual and social aspects. In this learning process, they base on their emotion, attitude, perception, and belief about what they can be observed. Especially, they prefer to learn from doing what they have believed rather than seeing or listening. Hence, transformative learning enables mankind to change their perceptions and beliefs in order to improve their community in positive and sustainable ways.

In the agricultural field, farmers can be considered as transformative learners because they need to acquire new and better understanding on changing their livelihood as well as improving their environment. In this study, T-Teachers (Transformative Teachers) consist of experts and scientists who can provide their community with more reliable and helpful information about environment and economy. Stakeholders are those who are local governments, functional officers in agricultural fields and other local unions. It is stressed that to form and develop transformative learning in a rural economy, people must actively share their knowledge and experience (Percy, 2005) as well as learn from other farmers' practical activities (Taylor and Cranton, 2012).

1.2 The VACB livelihood model

1.2.1 Livelihood

Livelihood is a concept used in many different forms and levels. It can be understood that "Livelihoods include capacity, assets, approaches (reserves, resources, ownership, usage rights) and activities which are necessary for human life" (Robert, 1983). In the DFID's (Department for International Development) Sustainable Livelihood Analysis Framework, "Livelihoods include capabilities, assets (including physical and social resources) and activities that are essential for living" (DFID, 1999). According to Tim et al (2004), a livelihood can be considered as a sustainable model when it is responsive and resilient with other impacts, or it can foster human abilities and assets in developing economy at the present and in the future as well as does not undermine the foundations of natural resources. In other aspect, Koos (2000) explains that a livelihood must depend on the possibilities and possessions (both material and social resources) and activities that are necessary for earning. The livelihood can be sustainable when it is able to support people coping with and recovering from any impact, and it can also accumulate or enhance human possessions as well as does not damage on natural environment (Hanstad *et al.*, 2004).

1.2.2 VACB

In this study, the livelihood of VACB can be considered as a sustainable livelihood model because it basically meets the livelihood needs of Phong Dien community. In other words, VACB model not only helps local communities to respond to climate change but also ensures human income as well as protects living environment. Specifically, this method of cultivation includes V is orchard (fruit, vegetable), A is the pond (freshwater fish), C is livestock (livestock and poultry) and B is Biogas (Gas is used in the family). It can be asserted that VACB livelihood model is understood as ways in which people earn their living in Phong Dien district, Can Tho city. Specifically, farming

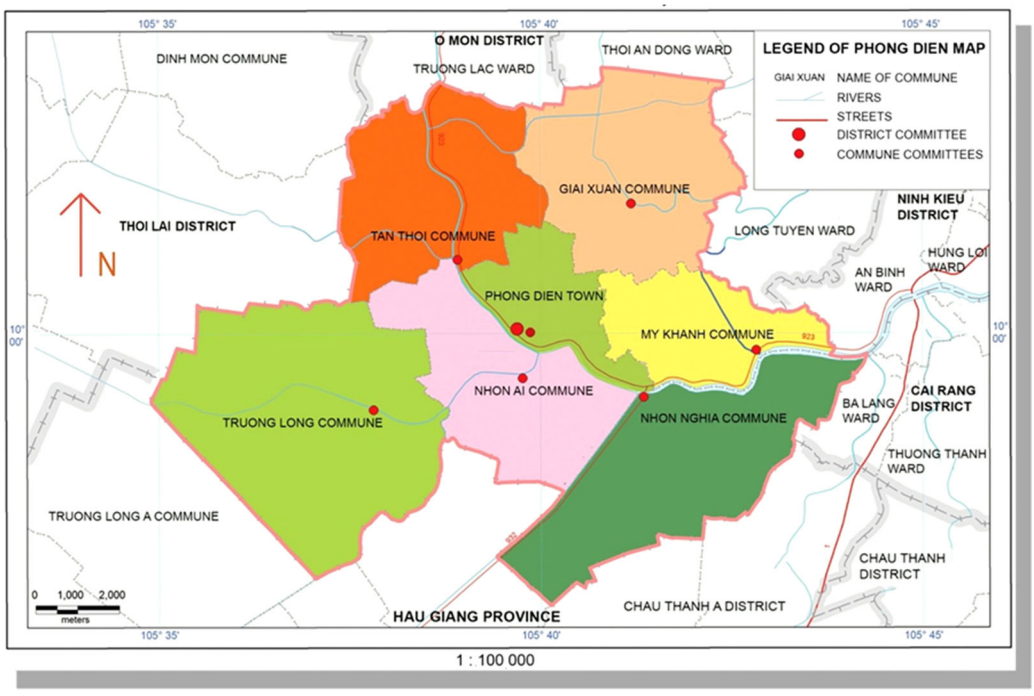
activities in Phong Dien include rice and fruit planting, animal husbandry, natural resource exploration, etc.

2 THE INTRODUCTION TO PHONG DIEN DISTRICT

Phong Dien is a suburb district belonging to Can Tho city located in the Mekong Delta region of Vietnam. In general, natural resources such as land, water, habitat, and climate provide Phong Dien with special potentials in developing agricultural economy (Nguyen Huu Chiem, 2012).

Environmental conditions in the study area have changed due to many reasons such as farming and living activities. This happened in a long time, so it caused a lot of harmful problems to the local life and economies. Specifically, more agricultural chemicals and wastes have been released into environment directly (Nguyen Huu Chiem, 2012). Based on residents' judgment, climatic variability has created a lot of changes such as erratic precipitation, hot weather, and more droughts. Those cause challenges to farmers in terms of their life and agricultural economics (Bui Thi Nga, 2009) as it is hard for the residents to adapt to huge changes in climate. Sometimes, the creatures are endangered or grow slowly and abnormally. Therefore, it requires conducting transformative learning in order to provide people with more useful information and experience amongst farming households. Firstly, this supports farmers to obtain more knowledge about the environment and environmental change, especially climate change. Secondly, farming households can share their experiences with other people in developing appropriate livelihood models in their local land.

However, during the time that transformative learning can be employed in Phong Dien, there were a lot of difficulties in maintaining transformative learning forms effectively in relation to residents' awareness and aptitude, local governments' support and information limitation. This makes local farmers confused with choosing the way to continue their transformative learning. Especially, the local people have not satisfied with transformative learning's outcomes. Due to this current situation, clarifying reasons and proposing solutions play an important role in order to develop appropriate climate – resilient livelihood models in Phong Dien district.



ADMINISTRATIVE MAP OF PHONG DIEN DISTRICT, CAN THO

Fig. 1: Administrative map of Phong Dien district

Source: Designed by authors, 2017

3 RESEARCH METHODOLOGY

3.1 Research questions

a. How was the VACB livelihood model formed?

b. What were difficulties in implementing transformative learning in climate-resilient VACB model in Phong Dien district?

c. What were the solutions to maintain and promote transformative learning in climate-resilient VACB model in Phong Dien district?

3.2 Data collection

Truong Long, Nhon Nghia and My Khanh communes were selected as research areas because the VACB model remains popular. In addition, there was different in the number of farmer households who are still implementing VACB model. In other words, current situation in conducting VACB model in these communes were not similar in outcome, technology and devise. With three different areas as above, this has supported the authors to gather reliable and diverse information.

In this study, mix-method was employed because it is believed that this can enable the researchers to obtain a profound research results due to diverse and helpful data. The respondents in this study are farmers, local leaders, university lecturers who used to take part in forming and developing VACB model in Phong Dien district. Thus, it is asserted that those respondents can provide the authors with rich and meaning full information.

3.2.1 Documentary study methods

In order to have a theoretical and practical basis for this research, academic documents in the field were collected and studied. Specifically, the collecting resources related to some different issues such as transformative learning (definition, components, conditions, and typical forms), climate change, (definition, expressions, causes and effects), livelihood and VACB model. The researchers have obtained the basic information about those issues from international and national books, journals, magazines, scientific yearbooks, and online forums which provided the authors with extensive and profound information. It cannot be denied that such understanding helped the researchers to approach research object easier.

3.2.2 Practical research methods Survey

In order to collect data for the study, the author have selected survey as a main data collection type which supported to collect a wide range information from surveying 40 households in three different communes consisting of Nhon Nghia, My Khanh and Truong Long. It is believed that the survey helped the researchers to gather broad and comprehensive information as the survey subjects were in different ages, races, incomes, academic background, occupations, etc.

Interview

To collect profound information as well as increase the reliability for the research, in-depth interviews have been selected. Through this data collecting way, the study have selected nine different interviewing subjects including experts, leaders and representative farmers in My Khanh commune and Phong Dien district. Those subjects were in different occupations, ages and experience; therefore, they have provided the authors with comprehensive and diverse information. This can support to improve the study results' reliability.

Expert observation and discussion

It is asserted that experts are those who understand very well about the development of VACB model adapting to climate change as well as transformative learning through this model. This explains why expert observation and discussion have considered as an essential data collection in the study. The research group directly worked and discussed with some experts in the field to gather information on the following issues: the climate change and environment in the selected area, formation and existence of VACB model, local residents' reactions to adapt to climate change, and transformative learning in VACB model in Phong Dien district. Through observing and discussing with experts, the authors have obtained a lot of reliable and valuable information in assessing the transformative learning process in climate change – resilient VACB model in Phong Dien district

Focus group discussion

In order to gather deep and highly critical information, the authors have carried out focus a group discussion. In this case, at the same time 34 participants have discussed about specified issues related to research topic. This was organized that 34 participants were divided into small groups of 4 to 6 people in each group. The researchers have moved around to support and orient participants to discuss about mentioned issues. When it was necessary, the researchers have suggested content or questions for participants in order to give their perspectives. After discussing in small groups, they shared their understanding, perspectives as well as experience on the issues. In this focus group discussion, participants

were those who are experts, local leaders and farmers. Thus, the focus group discussion provided the researchers with highly reliable data for this study.

3.3 Data recording

In order to collect, store and prove the research results, a number of data collection tools such as notebooks, recorders, cameras and telephones were used in this study. It is true that the questionnaires and interview questions were important to guide the data collection as well as to assist the authors collecting comprehensive information. Notebooks, cameras, voice recorders and telephones were tools that have helped the researchers to store collected data.

3.4 Data analysis

In order to analyze the collected information, specialized software such as SPSS (Statistical Package for the Social Science), Photoshop (image editing software) and MapInfo (mapping software) have been used. In particular, SPSS supported the authors to revise information systematically, put data into different themes and explain the research results. Photoshop or MapInfo has assisted the researchers in converting collected information into charts, maps, and datasheets. Specifically, the author used the Descriptive Statistics / Frequencies command to import, analyze data, calculate statistics parameters as a percentage description and verify them through Compare Means / Independent - Samples T Test commands. After that, the authors aggregated data and presented it in a tabular form. The analysis of data from SPSS software has provided the researchers with important and necessary information to create a practical basis for this study.

The researchers based on following process and technique in order to analyze collected data:

Step 1: Prepared and organized the data;

Step 2: Read through all data of interviews;

Step 3: Organized the material into segments of text before interpreting the meaning of data;

Step 4: Coded the data based on the meaningful segments and sort them into some different categories;

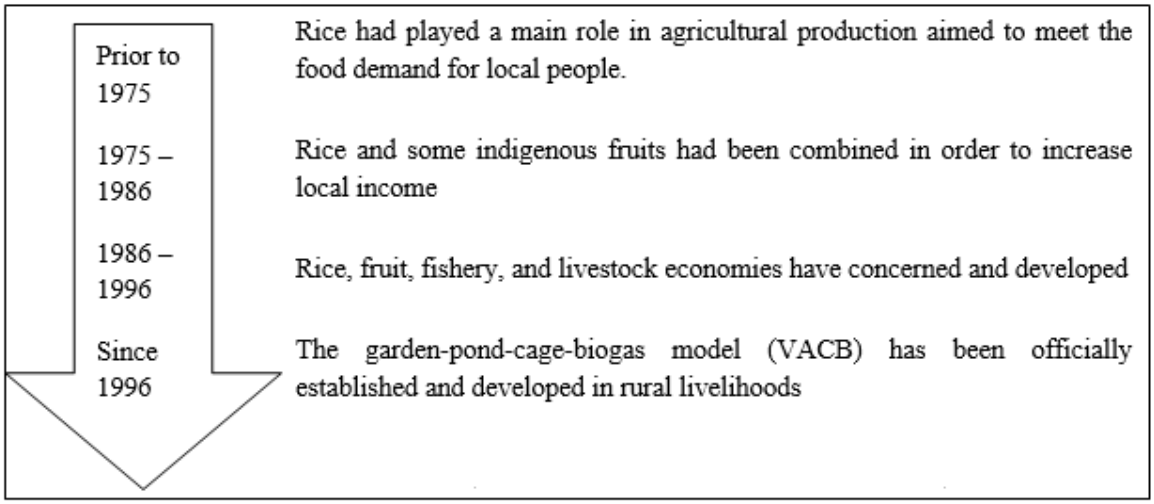
Step 5: Described the theme with typical meaning of each data sort. The researchers have focused on the main message of the category in each description;

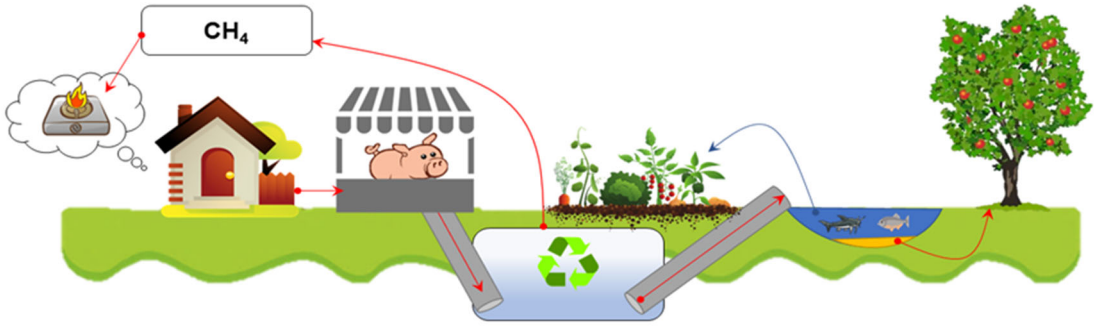
Step 6: Analyzed and concluded on the research results.

4 FINDINGS AND DISCUSSIONS

4.1 The process of changing livelihood in Phong Dien

According to data collected from expert observation and discussion, it was found that the livelihood in Phong Dien changed significantly. In general, the livelihood change in Phong Dien is summarized as follows:





Fig, 2: VACB model

Table 1: Biogas distribution in Phong Dien district in 2016

Commune/town	The number of households with Biogas
Phong Dien	29
My Khanh	31
Nhon Ai	34
Giai Xuan	57
Tan Thoi	61
Nhon Nghia	75
Truong Long	169

Survey results in October 2017

4.2 Forms of transformative learning in Phong Dien

To maintain and develop the sustainable livelihood model of VACB, some local people gradually changed their way of thinking, living in an open, progressive, practical and creative way. In other words, they have approached information and engaged in a variety of community-based activities to share information and experiences that were accumulated. The research results showed that the transformative learning in Phong Dien was conducted through seven different forms including self-learning, learning through workshops, learning through training, learning through model observation, learning through community activities, learning through media tools and learning through picking up (Figure 3).

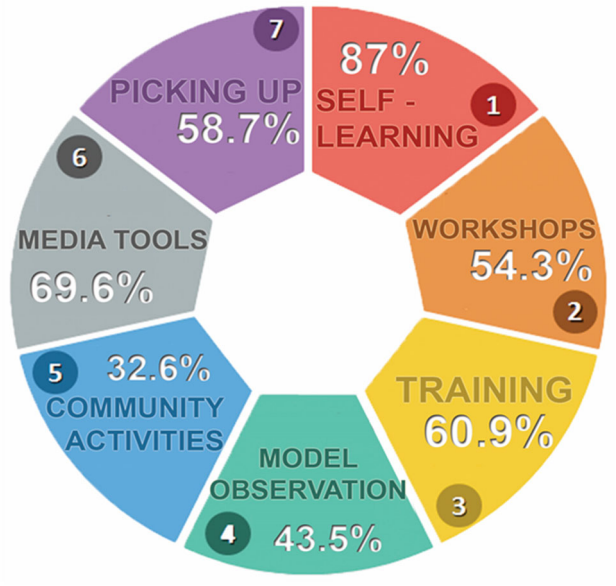


Fig. 3: Some forms of learning transfer in Phong Dien

Survey results of the study, n = 40 (October 2017)

The research results have shown that each form of transformative learning has its own characteristics and role in the local learning and sharing.

4.2.1 Self-learning

In the forms of transformative learning, self-study and self-reflection are more concerned with farmers

(87.0%). Since farmers find it difficult to believe other people and their thinking is less open, they tend to learn through self-observation and self-reflection. The process of self-observation and self-reflection is often based on practicality. In other words, they are hesitant to approach obscure and theoretical knowledge; instead, what can be observed and practiced is attractive to the local community. After the observation period, farmers try to get and check out results with what they have observed. At the workshop on Community Learning and Sustainable Livelihoods to Response to Climate Change in Can Tho City on October 12, 2017, Nguyen Van Hung (a farmer in My Khanh commune) shared "*To earn for our living as well as adapt to climate change, we have to learn from ourselves. We must save ourselves before someone else helps us"*.

It can be said that some farmers in this Phong Dien had a positive and forward thinking in coping with difficult problems. It cannot be denied that self-learning is a habit that is consistent with the cognitive characteristics of farmers in Phong Dien. In addition, rural lifestyles make farmers afraid to come and study in academic environments. This thinking is also appropriate because learning is to develop adaptive farming practices that require Phong Dien farmers to embark on practical things. In previous study, Percy (2005) concluded that in transformative learning process, people need to not only study themselves but also connect with other peer learning in order to exchange information and their practice actively. It can be implied that farmers' self-study activities in Phong Dien should be combined with other forms of learning.

4.2.2 Learning through workshops

In order to supplement knowledge and share experiences accumulated in rural livelihood development, workshops are opened by local authorities with institutes, schools, departments, international organizations, businesses, etc. In these workshops, besides listening, farmers also share and contribute their voices to leaders and businessman. This activity forms information sharing system among farmers and with others in their learning space. The research results indicated that workshops attract farmers to study at a rate of 54.3%. Mr. Nguyen Van Binh (a farmer in My Khanh commune) said, *"Thanks to the cooperation between local governments and other associations, organizations, and experts, we can learn from each other, especially we learn from people who have new knowledgeable and practical experience such as experts and lecturers. So I better understand about what climate change is and how to develop sustainable livelihoods model in order to respond to climate change"*.

According to Taylor and Cranton (2012), seminars and workshops need to be organized to attract stakeholders in the transformative learning process as stakeholders can provide people with useful and practical information. In this circumstance, it is true that opening workshops is to provide local people with basic scientific and necessary information. It is important for farmers to improve their understanding about the natural environment in general and climate change in specific. It can be said that when local people can know about their locality well in terms of natural conditions and potentials, they are able to develop their livelihoods effectively and efficiently.

4.2.3 Learning through training

Similarly, transformative learning through training is also attended by a large number of households (60.9%) due to constraints of local community management and education. In addition, the training is attended by many farmers as it responded well to practical needs of local livelihoods. For instance, training sessions usually introduce new livelihood models, practice advanced cultivation methods or experience which can help farmers to better adapt to climate change. According to Le Hoang Thanh (a farmer in My Khanh commune), *"Learning through training has somewhat escaped vague and abstract knowledge; therefore, I am interested in this learning form"*. So many farmers in Phong Dien appreciate and enthusiastically support to maintain and develop this form of transformative learning.

From survey and interview results, it is asserted that as transformative learning through training is not taking place frequently, so they did not have enough chances to learning and practice in applying new farming models. Besides, those training activities are usually organized in certain locations; therefore, local farmers sometimes find it hard to join in. In the future, it is suggested that local government should pay more attention to how to open more training courses in different localities. However, training will cost a lot of money and effort, so local authorities and agencies need to consider and prepare carefully to achieve the expected effectiveness. Especially, there should be a qualified and experienced workforce in organizing and conducting training (Mezirow and Taylor, 2009).

4.2.4 Learning through model-observation

Transformative learning in Phong Dien is often associated with the introduction of new models to adapt to climate change, so the form of learning through model-observation is also attractive to local human. However, this type of learning is not very common as there are only 43.5% of households who

participate in. Specifically, farmers can visit Biogas or VACB livelihood model which is made by representative farmers. They come to neighboring areas to observe, learn and exchange with other farmers in order to understand how to implement sustainable livelihood models. Combined with training and self-learning information, farmers try to do on their own agricultural economy. Mr. Cao Van Hai (a farmer in Truong Long commune) said that *"There are few models that can support local people to maintain their climate-resilient agricultural activities; however, some of such models have not worked effectively. So I do not have enough opportunities to learn through observing typical models"*.

It is sincere to state that although this form of learning is not common due to complex and inconvenient space and time, it provides people with more chances to learn authentically. In particular, many farmers can see and learn useful methods as well as check out results of typical livelihood models. Thus, this type of learning is helpful and reliable so that some farmers believe in and follow. Although there were no previous studies that demonstrate how model learning is useful, we can also acknowledged that learning through model is one of the most practical forms of learning. Therefore, local authorities need to create more conditions for farmers to study in this form.

4.2.5 Learning through community activities

Learning through community activities is less common due to difficulties of sharing information and changing farmers' perceptions and belief. In particular, there are only 32.6% farmers who usually participate in this study. The reason is that peasants find it hard to accept other perspectives, especially accepting unverified theories. Farmers argue that the contents of community activities are theoretically insufficient and are not attached to the need for livelihood development, so they do not want to participate in. In addition, the dispersion of habitat, the inconvenience of traffic, the difference in leisure time, etc. are major obstacles to this form of learning. Pham Dieu Linh (a member of the Women's Union of My Khanh commune) said "*It is true that Phong Dien district is one of rural areas with difficult conditions, especially about infrastructure and information technology equipment. Therefore, accessing and transferring information to farmers through community activities are limited*".

According to the study findings, Phong Dien does not have diversified activities in the community learning centers or spaces. Furthermore, community activities take place with lack of practical and updated information to suit real situations in developing livelihoods. They were reasons why some farmers do not want to join in community learning task. To form and promote helpful community activities, it is strongly believed that local authorities should coordinate with other organizations to design and organize activities that can provide local residents with more practical and necessary knowledge and practice. Based on Mezirow and Taylor (2009), the human transformative learning process only takes place when they are exposed and exchanged in their communities. From this point of view, it can be stated that Phong Dien farmers must inevitably participate in community activities to learn from each other and share their experiences in agricultural cultivation. It is true that farmers themselves need to change their thinking and opinions to receive and trust others in a critical way (Mezirow, 1997; Nguyen Duc Ngu, 2008).

4.2.6 Learning through media tools

Learning through media is useful to many farmers at the rate of 69.9%. This type of study is popular because of abundant free time in rural areas and the habit of accessing information associated with television programs is quite common. Mr. Vo Hoang Nam (a farmer in Nhon Nghia commune) said "*Watching television is convenient because it does not have to take time and effort to obtain reliable information, so every day I and my family spend time on watching television*". Starting from their own needs, people are often interested in reports or programs related to the local livelihood. Thanks to this, they can learn and enrich their understanding in order to develop their family's livelihood.

In fact, because technology equipment such as radio or loudspeaker is limited in many learning activities in the community, the use of television for both entertainment and study is essential. Once the basic information is obtained, the farmer can meet and discuss about what they have acquired in order to better understand. The result is that local farmers can share what they have known about farming works as well as livelihood models. Taylor and Cranton (2012) asserted that one of essential conditions that influence transformative learning is information technology. This implies that improving the media tools in Phong Dien district will support people to learn and share information more effectively. Therefore, the local and national government needs need to pay more attention to this if they want to promote transformative learning process quickly and effectively.

4.2.7 Learning through picking up

It is interesting that many households view learning through picking up as a good and effective way of

learning (58.7%). They have habits in observing and contemplating activities from other families or communities. After that, they try to do on their own economy so that they can build up their beliefs and change their mind. Based on Le Hoang Thanh (a farmer in My Khanh commune), *"I learn by this way because I can observe and verify results from other farmers"*. This implies that if farmers do not observe in real situations, it will be difficult for them to believe and follow.

The interview results have shown that learning through picking up is carried out purposefully because it just take place when farmers need to learn about livelihoods. Such learning form helps farmers to be self-aware and active in learning flexibly according to their needs, so they save time and are not bored during the learning process. Although no research has concluded that learning through picking up can supports residents to study well in transformative learning, the study showed its role in learning and sharing experiences to farmers in Phong Dien. Therefore, this form of learning should also be maintained but local authorities and stakeholders should supervise and participate in such learning activities in order to support local people. That will help farmers to orient content and learning methods in order to acquire good learning results.

4.3 Obstacles of implementing transformative learning in Phong Dien

4.3.1 Some difficulties on implementing transformative learning in Phong Dien

The research results have shown that there were some difficulties in implementing transformative learning as mentioned in below table:

Table 2: Difficulties on implementing transformative learning in Phong Dien

Difficulties	The rate of households facing difficulties (%)
Farmers improve spontaneously	69.6
Lack of government support	63.0
Lack of awareness	58.7
Distributed learning space	41.0
Lack of access to information	37.0
Limitation of information sharing policy	34.8
Short capacity	30.4

Survey results of the research team in October 2017

Farmers improve spontaneously

The spontaneous improvement of farmers without planning is a limitation for transformative learning in Phong Dien district, and 69.6% of farmers have such comments. According to Mezirow and Taylor (2009), transformative learning needs cohesion and exchange information and experience between individuals and groups or amongst people in the society. Spontaneity in learning and experiencing livelihood activities makes Phong Dien farmers often difficult and easy to fail. In addition, spontaneity makes it difficult to organize, manage, and deploy. Spontaneity is characteristic of working principles that are ancient and backward in Phong Dien. This also affects learning process as well as livelihood changing in this local community in some negative ways. Thus, it needs to concern about how to make plan to guide local residents.

Lack of government support

There are 63.0% of farmers saying that support from local authorities is still limited, so this becomes a big concern in this case. The study results have shown that farmers do not receive much support from the government and local authorities in learning and exchanging information. Especially, under uncertain market conditions and rapid ecological changes, people cannot do anything in some cases because of lack of scientific and orthodox information. According to Taylor and Taylor (2012), in the process of transformative learning, stakeholders play an important role because they are one of key players in this learning process. Specifically, it is roles of local government, women's union, organizations, enterprises. They need to support people in providing knowledge, directing and organizing, facilitating the connection among scientists, experts and farmers. However, this support is still limited, the transformative learning and maintenance of livelihoods have certain obstacles.

Limitation of awareness

Some farmers argue that their perceptions are limited, so this is a major obstacle to the implementation of transformative learning. In specific, 58.7% of people agree that weak awareness hinders the development of community learning. Local farmers do not have a basic understanding of transformative learning and its roles in improving knowledge and developing farmer livelihoods. When awareness is limited, people do not participate in some learning activities. Mezirow (1997) states that in order to learn effectively, learners need to be more aware of transformative learning, and then they can gradually improve their perceptions of what they need to do. From this point of view, it can be concluded that limited awareness is a real barrier of transformative learning in Phong Dien.

Distributed learning space

The results have indicated that 41.0% of farmers have difficulty because of distributed learning space. It can be seen that living space and scattered cultivation make transformative learning space of the community impossible to concentrate. In other words, when the conditions of travel are more inconvenient, and leisure time is also very different between households, local farmers find it hard to take part in any community work. Space dispersion does not create conditions for people to connect and exchange, so transformative learning is also hard to be took place (Taylor and Cranton, 2012). One of factors influencing transformative learning process is space because even large learning spaces need to be focused. However, these criteria in Phong Dien are incomplete; therefore, this impacts on farmer learning.

Lack of access to information

As indicated, 37.0% of farmers realized the means of accessing information in the area is lacking, so they do not have enough information. Some farmers find that there are no local loudspeakers. Moreover, radio stations in the locality present information that is outdated. In addition, many farmers have a low income, so they cannot buy computers, phones with network connection which can support them to update knowledge. Therefore, in order to obtain this information, people often learn by themselves thanks to watching television, talking with neighbors or taking part in seminars or training. Mezirow and Taylor (2009) mention that in today's era, leveraging the achievements of science and technology in transformative learning is a right move. Thus, it can be deduced that the limitations of media system hinder the process of changing people's perceptions, beliefs and actions.

Limitation of information sharing policy

The information sharing policy is also another issue discussed by some farmers (34.8%). They argue that local authorities do not have good policies to share information properly and effectively. Often, information from the state or university is disseminated to the local government quite quickly and efficiently through workshops, training or official documents. However, local authorities do not have effective ways to bring information to farmers. In some cases, local leaders also have no way to help people sharing information with each other. In some situations, information shared between farmers is outdated, which does not guarantee practical value.

Short capacity

There were 30.4% of farmers complaining about the capacity of representative farmers who play a pivotal role in transformative learning. They argue that some farmers do not have a good knowledge, so information can be shared is not correct or lack of scientific basis. Due to capacity limitations, some representative farmers must rely on their own subjective thinking. On the other hand, sometimes unproven personal experience has been shared broadly. In that case, inaccurate knowledge and experience is spread rapidly among farmers. It can be concluded that the limitations of knowledge or experience impedes the transformative learning in Phong Dien.

4.3.2 Solutions to the problems

To overcome obstacles in learning and sharing information, many farmers actively explore and experiment with many different ways. The research interviews have shown that there were 4 ways that local residents have reacted to learn and share information in the climate change-resilient VACB model as follows:

Look to representative farmers

To overcome their difficulties, many farmers come to other people who are representative in the field in order to learn and improve their livelihoods. They said *"If you do not actively learn, nobody will come and save you. It means that we must save ourselves first"*. It is a totally positive and appropriate perception not only in transformative learning but also in the development of local livelihoods to adapt to climate change. Undeniably, climate change always occurs with different levels and manifestations, so transformative learning and changing livelihood are real needs in this case. Mezirow and Taylor (2009) and Taylor and Cranton (2012) noted that in transformative learning, it is important to realize potential and important learner who is called representative learner as they can support other peer learner in learning process. Therefore, it is useful and encouraging to find representative peasants to learn. Hence, farmers themselves have gone through a process of changing their mind and belief – important process in transformative learning.

Take full advantage of meeting

Some farmers take full advantage of meetings at parties, local markets or even family gatherings to share and talk about their livelihoods. In the culture of Phong Dien district in particular and Can Tho city or the Mekong Delta in general, the exchange of information at meetings is very common. For example, in birthday and wedding parties, farmers meet and discuss about issues in their living and doing

business. Through these activities, information and experience in livelihood development can be shared quickly. There is no doubt that the transmission of such information is appropriate to the actual situation of rural areas as it does not cost and leads to positive results of transformative learning. According to Mezirow and Taylor (2009), transformative learning process only takes place when people exchange and cooperate with others. In other words, those tasks enable them to share information and transfer practical experiences in relation to agricultural cultivation. So, farmers in Phong Dien should take full advantages of any meeting so that they can obtain better understanding in adapting to changing environment and doing their farming works.

Learn through watching television

Watching TV or listening to local radio stations is another way which can helps Phong Dien farmers to get more information on climate change and their livelihoods in response to climate change. Le Hoang Thanh (a farmer in My Khanh commune) said *"We all have a television which is an important mean for us to have more information and experience. So, whenever we have free time we just open the television while lying down in order to entertain as well as study"*. It can be said that this is a way that supports local farmers to learn and improve their awareness on the issues. Moreover, information from the media is often censored, so the reliability is high so that people do not need to worry about information source. In transformative learning, information technology supports people to acquire academic knowledge through media tools; therefore, learners need to base on such tools to improve their understanding (Taylor and Cranton, 2012).

Access to the internet and social network

In other perspectives, small number of interviewees said that the Internet and social network also help them to get and exchange information and experience although this is not common practice. Mr. Tran Van Khoi said *"Today, technology is more developed and applied, so I can access to the Internet and get more new things to learn"*. In some cases, farmers are weak at information technology skills or do not know how to use technology equipment; they can rely on the support of other younger people. It is believed that this is a good and open response in thinking that can help farmers to access or share information together. This also means that they can learn more about climate change and how to develop livelihoods in response to climate change.

As such, it can be asserted that transformative learning is an inherent difficulty. The good thing is that Phong Dien farmers do not stop thinking and working together in order to build up a community with shared information and experience. Although the demand for transformative learning has not really met, with what farmers and local government think and react is a bright spot in this case. It is hoped that Phong Dien community will be more active and positive in improving their understanding in order to respond to climate change as well as develop their livelihoods more effectively.

5 CONCLUSIONS

According to the research results, it can be concluded some main issues as follows:

– Transformative learning has been formed and developed in Phong Dien district for a long time due to the demand of learning and sharing information among local residents. This learning form has supported local people to obtain a lot of information about their livelihood as well as about climate change. Especially, transformative learning enabled Phong Dien's farmers to understanding about the climate change as well as how to change their livelihood in order to adapt to the change of climate and environment.

– In implementing transformative learning in Phong Dien district it still has emerged many difficulties that need to be solved. Such difficulties related to residents' low aptitude and awareness, limited government support, lack of information and learning space. It is noted that those difficult problems hindered transformative learning in Phong Dien.

– From the difficulties that farmers in Phong Dien have faced as well as from residents' understanding, a number of solutions were proposed such as promoting the roles of local representative farmers, taking advantages of meeting amongst farmers or between farmers and other stakeholders, observing the information from television, internet and social network.

To enhance and promote transformative learning, some recommendations are proposed as follows:

Maintain and improve the representative farmers' capacity so that they can help other farmers in both carrying out local livelihood model and transformative learning activities.

– Organize more seminars and workshops to link and share information and experiences among farmers as well as to express their opinions.

– Improve information sharing system and social network to support local people updating more useful scientific information.

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