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CONTENT

Isolation and characterization of antibacterial compounds from *Euphorbia tirucalli* against *Xanthomonas axonopodis* pv. *citri*

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ABSTRACT

Xanthomonas axonopodis pv. *citri* (*X. axonopodis* pv. *citri*) is the cause of canker disease on lime trees that negatively affect plant health and fruit quality. This study focused on the comparison of the extraction yield and antibacterial properties of *Euphorbia tirucalli* against *X. axonopodis* pv. *citri*, phytochemical screening, quantification of phenolic and flavonoid contents of the fraction extract. The results showed that the ethyl acetate fraction (EA) (7.5 mg/mL) of *E. tirucalli* from Dak Nong province had the best activity against bacteria with diameter of inhibition zone determined 15.50 ± 0.50 mm, and the minimum inhibition concentration was 0.312 mg/mL. Alkaloids, flavonoids, tannins, and terpenoids were found in the EA fraction extract of E. tirucalli, whereas saponin did not appear in the extract. The phenolic and flavonoid content was in the range of 14.46 - 98.63 mg GEA/g and 90.34 - 408.86 µg QE/g, respectively. Column chromatography followed Nuclear Magnetic Resonance spectra were performed and the three compounds were identified as scopoletin, gallic acid, and 3,3',4'-tri - O - methylellagic acid. This study suggests that the extract from E. tirucalli and the isolated compounds can be used for managing of citrus canker disease.

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1. Introduction

Xanthomonas axonopodis pv. *citri* causes citrus canker disease that commonly occurs in citrus-growing regions. Bacterial colonisation causes lesions on fruits, leaves, and stems of citrus trees, including lime, orange, and grapefruit (Jalan et al., 2013). Using chemicals is the best strategy for preventing diseases and insect pests, but the overuse of chemicals with their residual toxicity is causing serious health hazards to humans, animal life, and the environment. To reduce or avoid the harmful effects of synthetic chemicals, there is an urgent need to find alternative methods for managing plant pathogens. Plants have developed effectively defense systems that consist of several complex secondary compounds. Antimicrobial compounds in plant tissues are an essential factor in disease resistance (Fawcett & Spencer, 1970), and used as fungicides, pesticides, and bactericides (Dubey & Kishore, 1987; Radcliffe et al., 1991). The development of plant-based compounds for pest control is a priority interest in sustainable agricultural production.

Euphorbia tirucalli, known as "san ho xanh" or "xuong ca" in Vietnam, is a popular herb known worldwide for its multiple uses. Stem latex is used in treating abdominal pain, asthma, cough, earache, intestinal worms, leprosy, rheumatism, skin

diseases, toothache, tumors, and warts (Ramesh et al., 2009). Previous studies have shown that active compounds from this plant, such as alkaloids, phenols, and tannins, contribute to medicinal treatments (Sugumar et al., 2010). The bacterial activity of *E. tirucalli* has reported on *Erwinia carotovora, Fusarium oxysporum,* and *Xanthomonas campestris* (Lirio et al., 1998); *Alternaria alternata, Aspergillus flavus, Aureobasidium pullulans, Bacillus subtilis, Drechslera oryzae, Escherichia coli, Pseudomonas aeruginosa, P. solanacearum, Staphylococcus aureus, Xanthomonas citri* (Jadhav et al., 2010); *Aspergillus fumigatus, A. flavus, A. niger, Bacillus subtilis, Candida albicans, C. tropicalis, Escherichia coli, Fusarium oxysporum, Klebsiella pneumoniae, Pseudomonas aeruginosa, Proteus vulgaris, Salmonella enteritidis,* and *Staphylococcus aureus* (Upadhyay et al., 2010). *Euphorbia tirucalli* is a good example, but little information is available on plant diseases, especially against phytopathogens in Vietnam. Therefore, this study was conducted on *E. tirucalli* with the following objectives: (i) to screen and evaluate the antibacterial activity of fraction extracts (hexan, ethyl acetate, and butanol) against *Xanthomonas axonopodis* pv. *citri*; (ii) to isolate and identify the bioactive compounds using Thin Layer Chromatography (TLC) analysis, Column Chromatography, and Nuclear Magnetic Resonance (NMR) spectra.

2. Materials and Methods

2.1. Biological materials

The whole fresh aerial parts of *E. tirucalli* (from two to four years old) was obtained from Dak Nong province (DNP) – MG (12°15'25" North latitude 107°42'06" East longitude) and Ho Chi Minh City (HCMC) – MG (10°48' North latitude 106°39' East longitude) on April, 2019.

The bacterial *X. axonopodis* pv. *citri* strain BLKQ1 (AN: MT328595) was isolated from canker disease on lime trees and preserved in Faculty of Biological Sciences, Nong Lam University Ho Chi Minh City, Vietnam.

2.2. Preparation of *E. tirucalli* extracts

Plant samples were thoroughly washed under running tap water, air-dried and subsequently dried in a hot air oven at 60°C for 72 h. Then, dried samples were milled to obtain 6.5 kg powder. The powder was drenched with 96% ethanol (EtOH) (5 × 15 L) for 24 h, to obtain the EtOH - soluble extract. After filtering, the EtOH - soluble was evaporated to obtain the crude ethanol extract. The ethanol extract was sequentially partitioned with n - hexane, ethyl acetate, and butanol to obtain the respective fractions (He, EA, and Bu). The percentage yield (%) was calculated as the ratio of the weight of the extract to the dried weight of the sample.

2.3. Antibacterial assay

The antibacterial properties of the fraction extracts were evaluated using the agar well diffusion method (Toda et al., 1989). Each fraction extract was dissolved in 10% dimethyl sulfoxide (DMSO), reaching final concentrations of 1.25, 2.5, 5.0, and 7.5 mg/mL for further testing (Upadhyay et al., 2010). Bacteria were cultured in nutrient broth (NB) for 20 - 24 h at 37°C and adjusting of the concentration to 0.5 McFarland Standard with sterile NB. Wells of 5.0 mm were punched in the agar medium and were filled with 60 µL of fraction extract. Streptomycin (0.01 mg/mL) was used as the positive control while 10% DMSO was used as a negative control. Inhibition zones (mm) were measured after 48 h of incubation at 37°C. Each assay was performed in triplicate.

2.4. Minimum inhibitory concentrations assay

Minimum inhibitory concentrations (MICs) were measured following the recommendations of the NCCLS (2003), and Devienne & Raddi (2002). Each extract was diluted to 5.0 - 0.078 mg/mL by dispensing 50 µL of extracts into each of the 96 wells of a standard microtiter tray containing 100 µL of Nutrient Broth followed by 2-fold dilutions. Bacterial cultures (100 µL) were added to each well. After bacterial growth, 20 µL resazurin (0.01%) was added to each well and incubated at 37°C for 2 h. The MIC was determined visually as the lowest concentration that led to growth inhibition after 2 h.

2.5. Phytochemical Screening

The phytochemical screening was performed according to Brain & Turner (1975) and Evans (1996) with minor modifications.

2.5.1. Alkaloids

H_2SO_4 5% (20.0 mL) was added to 2.0 g of fraction extract and filtered. Then, a few drops of NH3 were added to the filtrates until became alkaline. Chloroform (5.0 mL) was added for separating the aqueous and organic layers. Then, 2.0 mL of 1% HCl and 1 mL of Wagner's reagents were added to the organic layer. There was a brown/reddish - brown formation, indicating the presence of alkaloids.

2.5.2. Flavonoids

Ethanol 70% (20 mL) was added to 5 g of fraction extract and heated to boiling. After filtering, a few drops of H_2SO_4 were added to the solution. There was an orange colour formation, indicating the presence of flavonoids.

2.5.3. Saponins

About 10 mL of distilled water was added to 1 g of fraction extract, boiled, and cooled. The solution was vortexed for 15 sec. There was no foam formation, indicating the presence of saponins.

2.5.4. Tannin

Distilled water (100 mL) was added to 10 g of fraction extract. The solution was heated in a water bath for 3 min, cooled, and filtered. The ferric chloride was added to the solution. There was a dark green colour formation, indicating the presence of tannin.

2.5.5. Terpenoids

$CHCl_3$ (2 mL) and concentrated H_2SO_4 (3 mL) were added to 5 mg of fraction extracts. There was a reddish-brown colour formation in the inner face, indicating the presence of terpenoids.

2.6. Quantification of phenolic content

The total phenolic content of the extract was measured according to the method of Waterman & Mole (1994) using a UV - Vis spectrophotometer (Jenway 6100, Dunmow, Essex, U.K). First, either 0.5 mL of each extract or an appropriate gallic acid standard was added to 2.5 mL of 1/20 diluted Folin reagent and mixed for 4 min, after which 2 mL of 10% Na_2CO_3 solution was added and mixed. After standing 30 min and shaking, the absorbance of the mixture was measured at 758 nm. Total phenolic was expressed as milligrams of gallic acid equivalents per gram of sample (mg GAE/g of the extract). All samples were analysed in triplicate.

2.7. Quantification of flavonoids content

The total flavonoid content of the extract was measured according to the method of Zhishen et al. (1999) using quercetin as a standard. Either 1 mL of each extract or an appropriate quercetin standard was added to 4 mL of distilled water, after which 0.3 mL of 5% NaNO2 solution was added. The solution was added 0,3 mL of 10% $AlCl_3$ (w/v) and 2 mL of 1 M NaOH and produced a total volume of 10 mL with distilled water. After 30 min, the solution was measured in a UV - Visible Ultraspec JANEWAY 7305 spectrophotometer at 320 nm. The contents were expressed as microgram equivalents of quercetin per gram of extracts (µg QE/g of extract). All samples were analysed in triplicate.

2.8. Column chromatography and isolation

Ethyl acetate (EA) fraction of *E. tirucalli* from DNP was run onto the silica gel column and eluted with different concentrations of n - hexane, ethyl acetate, and acetone (in the proportion 5:1:1 to 1:1:1, v/v/v). A total of 8 fractions had been collected (20 mL each) and marked as EA1 - 8. The fraction EA3 was adsorbed on a silica gel column and eluted with different concentrations of n - hexane, chloroform, ethyl acetate, acetone, and acetic acid (in the proportion 350:100:40:25:10, v/v/v/v/v). A total of 4 fractions had been collected (20 mL each) and marked as EA3.1 - EA3.4. EA3.2 was adsorbed on the silica gel column and eluted with methanol, and collected three fractions EA3.2.1 - EA3.2.3. EA3.2.1 (97.0 mg) was run onto the silica gel column and eluted with methanol and water (in the proportion 1:2 to 1:1, v/v). A total of three compounds had been collected and named as 1 (12 mg), 2 (3.7 mg), and 3 (7.6 mg).

2.9. The NMR profiling of compounds

The NMR spectra were recorded in CD_3COCD_3 on Bruker Avance III spectrometers at 500 MHz (1H) and 125 MHz (13C). The

signals of the residual solvents of CD_3COCD_3 at δH 2.05 and δC 29.4 were taken as reference points. The NMR temperature was set at 23°C.

2.10. Statistical analysis

The data were analysed using SPSS 20.0, where ANOVA (one way) or t-tests were used to show the statistical difference at a 95% confidence. The results were further subjected to Duncan test to separate the significantly different means.

3. Results and Discussion

3.1. Extraction yield

The extraction yield of the crude extract (EtOH) and fraction extracts (He, EA, and Bu) of *E. tirucalli* from DNP and HCM are given in Table 1. The extraction yield varied among the different solvents and regions to collect plants. The EtOH extract of sample from DNP showed a higher extraction yield of that from HCM. Compared to other fractions and regions obtained, *E. tirucalli* from DNP showed the extraction yield of EA fraction was highest (18.34%) while *E. tirucalli* from HCM showed that the extraction yield of n - hexan and Bu fractions were highest at 31.13% and 15.22%, respectively. This result showed that the composition and content of secondary compounds depended on geography, climate, age of trees (Upadhyay et al., 2010). According to Younes et al. (2018), the polarity and molecular weight of the chosen solvent effect phytochemical constituents in the extracts.

3.2. Antibacterial activity of fractions

Antibacterial activity of fractions The ability against bacteria of the fraction extracts from DNP and HCMC is presented in Table 2. At 5 mg/mL, all fractions exhibited antibacterial activity with inhibition zones ranging from 3.07 ± 0.60 to 12.23 ± 0.87 mm. The EA fraction (7.5 mg/mL) from DNP showed the highest inhibition diameter (15.50 ± 0.50 mm), and the MIC value was 0.312 mg/mL. Meanwhile, the He fraction (2.5 mg/mL and 1.25 mg/mL) from all two regions did not show antimicrobial activity. All fractions collected from DNP sample exhibited higher antibacterial activity than those obtained from the HCMC sample. The antimicrobial ability of EA fraction from DNP exhibited higher than those of neem and garlic extracts of Negi & Kuma (2015).

3.3. Phytochemical screening

The phytochemical composition of fraction extracts from DNP is shown in Table 3. Flavonoids are present in all fractions but any (not?) saponin, a toxic group. Alkaloids and tannins were positive in EA and Bu fractions, where terpenoids were positive in He and EA fractions. Compared to other fractions, EA fraction possessed more phytochemical constituents. Our results were similar to the report of Orlanda & Vale (2015). Controversy, Aleixo et al. (2018) reported that flavonoids present in the extract of *E. tirucalli* from Brazil, but not tannin, alkaloid, or saponin.

3.4. Phenolic and flavonoid content

The phenolic and flavonoid content of fraction extracts from DNP is shown in Table 4. Two linear equations ($y = 0.102x + 0.0075$, $R^2 = 0.9998$; $y = 0.0043x + 0.0118$, $R^2 = 0.9999$) were constructed based on the standardised gallic acid and quercetin amount used calculating the total phenolic and flavonoid content. All fractions of E. tirucalli from DNP contained varied phenolic and flavonoid content. Of the three fractions, EA has the highest phenolic and flavonoid content corresponding to 98.63 ± 4.14 mg of gallic acid equivalent (GAE)/g and 408.86 ± 7.67 µg quercetin equivalent (QE)/g. The lowest phenolic and flavonoid content were obtained with He, corresponding to 14.46 ± 1.53 mg GAE/g and 90.34 ± 2.90 µg QE/g. Besides chlorophyll and lipids, the He fraction also contained a few low polarity phenolic compounds (free-form flavonoids). The phenolic content of the EA fraction from DNP was higher than that of *E. tirucalli* from Brazil (Orlanda & Vale, 2015).

3.5. Antimicrobial compounds from *Euphorbia tirucalli* and its structure determination

Among fraction extracts, the EA fraction of DNP showed higher antibacterial activity was used to isolate antimicrobial compounds. The results of NMR showed the presence of scopoletin (Figure 1A), gallic acid (Figure 1B), and 3,3',4'-tri-O-methylellagic acid (Figure 1C). Data of NMR Spectra of three purified compounds are

Table 1. Extraction yield (%) by different solvents of *E. tirucalli* from Dak Nong province (DNP) and Ho Chi Minh City (HCMC)

No.	Solvents	Extraction yield (%)	
		DNP	HCMC
1	Ethanol (EtOH)	8.87 ± 0.05^{b}	8.67 ± 0.03^{a}
2	n-Hexan (He)	29.79 ± 0.30^{a}	31.13 ± 0.82^{b}
3	Ethyl acetate (EA)	18.34 ± 0.67^{b}	15.92 ± 1.07^{a}
4	Butanol (Bu)	13.14 ± 0.44^{a}	15.22 ± 0.57^{b}

[a-b]Values in the same row with different letters were significantly different at $P < 0.05$ (mean ± SD, n = 3).

Table 2. Antibacterial activity of fraction extracts of *E. tirucalli* from province (DNP) and Ho Chi Minh City (HCMC)

Sample	Fraction extracts	Inhibition zone diameter (mm)			
		Concentration (mg/mL)			
		1.25	2.5	5.0	7.5
DNP	n-Hexan (He)	-	-	3.07 ± 0.60	5.77 ± 0.75
	Ethyl acetate (EA)	8.67 ± 0.29	10.17 ± 0.29	12.23 ± 0.87	15.5 ± 0.50
	Butanol (Bu)	3.27 ± 0.25	5.00 ± 0.50	7.67 ± 0.76	10.03 ± 0.55
HCMC	n-Hexan (He)	-	-	2.67 ± 0.29	5.00 ± 0.50
	Ethyl acetate (EA)	6.67 ± 0.29	8.40 ± 0.36	9.97 ± 0.45	11.60 ± 0.36
	Butanol (Bu)	2.93 ± 0.40	4.67 ± 0.29	7.07 ± 0.60	8.33 ± 0.76
Streptomycin (0.01 mg/mL)		20.13 ± 0.61	20.13 ± 0.61	20.13 ± 0.61	20.13 ± 0.61
DMSO 10%		-	-	-	-

(mean ± SD, n = 3); "-" non-inhibition.

Table 3. Phytochemistry composition in fraction extracts of *E. tirucalli* from Dak Nong province

Fraction extracts	Alkaloids	Tannin	Saponin	Flavonoids	Terpenoid
n-hexan (He)	-	-	-	+	+
Ethyl acetate (EA)	++	+	-	++	+++
Butanol (Bu)	+	+	-	+	-

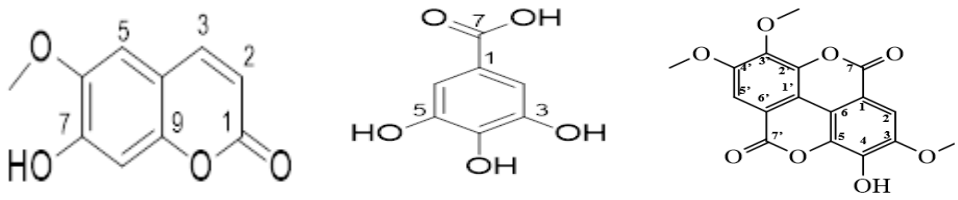
"-": negative; "+,++, and +++": Low, moderately present, highly present.

Table 4. Phenolic and flavonoid content in fraction extracts of *E. tirucalli* from Dak Nong province

Fraction extracts	Phenolic content (mg GEA/g)	Flavonoids content (µg QE/g)
n-hexan (He)	14.46 ± 1.53	90.34 ± 2.90
Ethyl acetate (EA)	98.63 ± 4.14	408.86 ± 7.67
Butanol (Bu)	50.94 ± 2.72	258.75 ± 2.82

shown in Table 5 and Table 6.

According to previous studies, scopoletin, gallic acid, and 3,3',4' - tri - O-methylellagic acid showed antimicrobial activity (Kuete et al., 2007). The mechanism of bacterial inhibition of scopoletin has been observed similar to antibiotic β - lactam. It inhibited cell wall formation and distorted bacterial morphology (Tiwtawat et al., 2018). For example, scopoletin inhibited Staphylococcus aureus (Natividad et al., 2019); *Salmonella typhimurium* (Wisnu & Risna, 2020); *Pseudomonas aeruginosa* (Tiwtawat et al., 2018); *Actinomyces naeslundii; A. israelii; Actinobacillus actinomycetemcomitans; Prevotella intermedia,* and *Porphyromonas gingivalis* (More et al., 2012). Gallic acid, a phenolic acid, against various bacteria such as *X. citri subsp. citri* (Silva et al., 2013); *Pseudomonas putida, P. fragile; P. fluorescens* (Elena et al., 2018); *Escherichia coli; P. aeruginosa, Staphylococcus aureus,* and *Listeria monocytogenes* (Anabela et al., 2013); *Stenotrophomonas maltophilia* (Navarro - Martinéz et al., 2005). According to Kuete et al. (2007), 3,3',4-Trimethylellagic acid from *Irvingia gabonensis* inhibited the growth of six Gram (+) and thirteen Gram (-) tested species. The results



(A) (B) (C)

Figure 1. (A) structure of Substance 1 (scopoletin); (B) structure of Substance 2 (gallic acid); (C) structure of Substance 3 (3,3',4'-tri-O-methylellagic acid).

Table 5. Data of NMR Spectroscopy of Substance 1 and Substance 2

Position	Substance 1 (Scopoletin)		Substance 2 (Gallic acid)	
	δ_H, J(Hz)	δC	δ_H, J(Hz)	δ_C
1				122.2
2		160.4	7.14 (1H, s)	110.4
3	6.17, d, 9.5	112.4		146.4
4	7.84, d, 9.5	143.7		139.6
5	7.19, s	109.1		146.4
6		144.9	7.14 (1H, s)	110.4
7		154.1		170.5
8	6.80, s	102.8		
9		150.9		
10		111.2		
11				
3-/5/6-OCH_3	3.90	55.7		
O-CH_2-O				

Table 6. Data of NMR Spectroscopy of Substance 3

Position	Type C	Substance 3		3,3',4'-tri-O-methylellagic acid	
		δ_H (ppm)	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)
1	>C<		111.70		111.62
2	>C<		140.91		141.23
3	>C<		140.71		140.26
4	>C<		153.68		152.23
5	-CH-	7.54 (1H, s)	107.38	7.73 (1H, s)	111.42
6	>C<		112.42		112.11
7	>C<		158.29		158.52
1'	>C<		111.78		111.65
2'	>C<		141.40		141.25
3'	>C<		140.22		140.23
4'	>C<		153.04		152.22
5'	-CH-	7.48 (1H, s)	107.38	7.61 (1H, s)	111.45
6'	>C<		113.34		112.12
7'	>C<		158.47		158.63
3-OMe	-CH_3	4.07 (3H, s)	61.25	4.19 (3H, s)	61.53
3'-OMe	-CH_3	4.06 (3H, s)	60.91	4.14 (3H, s)	61.26
4'-OMe	-CH_3	3.98 (3H, s)	56.66	4.04 (3H, s)	56.51

of this study provide the scientific basis to confirm the inhibitory activity of *X. axonopodis* pv. *citri* of EA extract of *E. tirucalli* from Dak Nong province.

4. Conclusions

The results showed that the extraction yield of sample from Dak Nong province is higher than that of Ho Chi Minh city. The phytochemical screening revealed that alkaloids, flavonoids, tannins, and terpenoids were in the ethyl acetate fraction, but not saponin in all fractional extracts. The ethyl acetate fraction produces higher amounts of phenolic and flavonoids and antimicrobial activity against Xanthomonas axonopodis than other fractions. From this fraction extract, scopoletin, gallic acid, and 3,3',4' - tri - O - methylellagic acid were identified.

Conflict of interest

The authors declare no conflict of interest.

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The effects of growing media on growth and seedling quality of root cutting of *Cyclea barbata* Miers at nursery stage

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ABSTRACT

Cyclea barbata Miers can be used in pharmaceutical industries and food. They are of tropical origin, and thus suitable for cultivation in many places in Vietnam. This study was conducted to evaluate the effects of different types of growing media on growth, seedling quality of root cutting of *Cyclea barbata* and financial efficiency at the nursery stage. A one-factor experiment was arranged in a completely randomized design with three replicates and nine treatments which were different mixtures of sand, rice husk ash, coco peat and vermicompost with different ratios in volume. The results showed that root cutting of *Cyclea barbata* was planted on the mixed media of 25% rice husk ash: 50% coco peat: 25% vermicompost gave the highest number of roots (17.23 roots/cutting), shoot length (100.48 cm), number of leaves (14.70 leaves/cutting), fresh and dry root matter (3.71 and 0.48 g/cutting), fresh and dry shoot matter (5.45 and 1.50 g/cutting), seedling quality index (0.019) at 75 days after planting, the profit (3,665,800 VND/1000 cuttings) and a profit margin of (0.73 times).

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1. Introduction

Cyclea barbata Miers is a plant species native to tropical climates, distributed in some Southeast Asian countries and India (Manilal & Sabu, 1985), so it is suitable for climatic conditions in many areas in Vietnam. Pham (2006) indicated that jelly made from *Cyclea barbata* leaves can lower body heat and diuretic; Root of *Cyclea barbata* can cure liver diseases, haemorrhoids. In addition, leaf jelly is also used in the food industry as a food thickener and stabilizer (Arkarapantth et al., 2005). Many southern provinces such as Binh Phuoc, Ba Ria - Vung Tau, and Dong Nai in which *Cyclea barbata* is grown.

Cyclea barbata is commonly propagated by seeds and root cuttings. Root cutting is an asexual propagation method that helps the plantlets to retain the desired characteristics of the mother plants. The root cutting technique of *Cyclea barbata* is simple, easy to implement and does not require any modern equipment. Many factors effect the quality of seedlings at the nursery stage. In particular, the growing media plays an important role in providing air and water, allowing optimal root development and responding to the physical properties of the plant (Olle et al., 2015). In addition, appropriate use of growing media also helps to make effective use of agricultural waste (Pham et al., 2018). However, each type of material used to mix the growing media has different characteristics, so it is necessary to determine the appropriate ratio of mixing media to help the cuttings grow well and achieve the best quality and

gain the highest economic benefit.

2. Materials and Methods

2.1. Experimental design

The experiment was carried out from July to September 2020 at the Experimental Station of the Faculty of Agronomy, Nong Lam University, Ho Chi Minh City.

The experiment was single-factorial and arranged in a Completely Randomized Design with 9 treatments corresponding to 9 types of media (denoted G) and 3 replicates. Nine types of media were described in Table 1.

The cuttings of *Cyclea barbata* root was 3 cm in length and 1 cm diameter. Each cutting was planted in a nylon grow bag contained 60 cm^3 of growing media. Each experiment plot included 30 cuttings planted in 30 grow bags.

2.2. Land preparation and field management

The ingredients were mixed by volumetric proportions corresponding to the formulated media. After 3 days of mixing and incubation, the compost would be supplemented with Trichoderma at a rate of 2 kg per m^3 and Super Phosphate at a rate of 3 kg per m^3. Then the mixture was mixed well every ten days for 30 days. The composts were covered using plastic film to prevent heat and nitrogen loss.

After 30 days, each type of media was sampled and analysed physical and chemical properties at the Department of Soil Science - Fertilizer, Faculty of Agriculture, Nong Lam University. The media properties are presented in Table 2.

Analytical methods include pH meter ($pH_{H_2O}(1:5)$), EC meter (EC (1 : 5)), Kjeldahl (total N), Tiurin (total organic), colorimeter (total P_2O_5), flame photometer (total K_2O), metal ring (bulk density), picnometer (density), drying method (humidity).

The results of Table 2 suggested that all media were slightly acidic but not salty (Slavich & Petterson, 1993). Total nitrogen content of the mixed media: G1 (50% sand + 25% rice husk ash + 25% coco peat (the control)); G2 (25% sand + 50% husk ash + 25% coco peat; G3 (25% sand + 25% husk ash + 50% coco peat); G4 (50% coco peat + 50% rice husk ash) were an average while the other media had high nitrogen content. Total phosphorus and potassium content of the media G1 (50% sand + 25% rice husk ash + 25% coco peat (the control)); G2 (25% sand + 50% husk ash + 25% coco peat); G3 (25% sand + 25% husk ash + 50% coco peat); G4 (50% rice husk ash + 50% coco peat) were relatively low, while the other media were high. All media had a high total organic content, ranging from 8.97 to 29.42%, and C/N ratio was from 16.44 to 60.58 (Rayment & Lyons, 2011). Besides, the media after incubation all had average porosity and moisture content. In general, the types of media were supplemented with vermicompost that had a higher content of macronutrients and porosity than the rest of the media.

Preparing the cuttings, planting and experiment managements: Root cuttings were harvested under the ground by carefully digging to avoid wounded inducing to the roots. Choose roots with a diameter of about 1.0 - 1.2 cm then cuttings without scratches, crushed on the outside into sections of about 3 cm long, then dipped in NAA solution the concentration of 1000 ppm in 2 sec for initiating rooting. After that, the 3 cm length cuttings were planted on sand and watered twice a day. After 20 days, the cuttings were transplanted into grow bag containing 20 cm^3 of media. Slow-release organic fertilizer (Bounce Back) was applied at a dose of 2 g/bag at 30 and 60 days after planting (DAPs). Two weeks after transplanted, all of the cuttings were watered twice a day in the early morning and late afternoon.

2.3. Data collection and statistics

During 75 days DAPs, data were measured from one-third of the cuttings (10 cuttings per plot).

Root number (roots/cutting): Count all of the main roots growing from the cutting; Root length (cm): Measure the length of main roots from the root neck to the longest point of the root; Shoot length (cm): Measure the length of shoot from the base of the trunk to the highest point of the shoot; Number of leaves/cutting (leaves/cutting): Count all of the leaves growing from the shoot; Chlorophyll Content Index (CCI): Use a chlorophyll meter (CCM-200 Plus) to measure at the center of the leaves.

Fresh roots matter (g/cutting): Weigh all the roots after removing the media.; Dry roots matter

Table 1. Nine types of media in the experiment

Symbol	Growing media
G1 (control)	50% sand + 25% rice husk ash + 25% coco peat
G2	25% sand + 50% rice husk ash + 25% coco peat
G3	25% sand + 25% rice husk ash + 50% coco peat
G4	50% rice husk ash + 50% coco peat
G5	50% rice husk ash + 50% vermicompost
G6	50% coco peat+ 50% vermicompost
G7	25% rice husk ash + 25% coco peat + 50% vermicompost
G8	25% rice husk ash + 50% coco peat + 25% vermicompost
G9	50% rice husk ash + 25% coco peat + 25% vermicompost

(g/cutting): Weigh all the roots after removed the media and dried at 70°C until the weight is constant; Fresh shoot matter (g/cutting): Weigh all the shoot; Dry shoot matter (g/cutting) (g/cut): Weigh all the shoot after dried at 70°C until the weight is constant.

Dickson Quality Index: DQI = Total dry matter/[(Shoot height/ stem base diameter) + (Dry shoot matter/Dry root matter)]; Percentage of live cuttings (%) = (Total number of live cuttings/30) × 100; Commercialarable ratio (%) = (Total qualified cuttings/30) × 100.

Economic efficiency: Total expenditure (VND/1000 cuttings): Cost of agricultural materials + electricity and water + labor; Total revenue (VND/1000 cuttings): Number of cuttings qualified x selling price; Profit (VND/1000 cuttings) = Total revenue minus total expenditure; Profit ratio (times) = Profit/Total expenditure.

The collected data were statistically calculated using Microsoft Excel, and analysed with ANOVA and Duncan's test at the significance level $\alpha = 0.05$ using the SAS 9.1 program.

3. Results and Discussions

3.1. Effect of growing media on root number and root length of *Cyclea barbata*

Different types of media have different physical and chemical properties, especially in terms of bulk density and porosity. Long (1993) stated that media plays an important role in the success of propagation by cuttings. The number of roots and root length of the cuttings at the time of 75 DAPs are presented in Table 3.

The results of Table 3 show that the number of roots on the cuttings is significantly different ($P = 0.01$) when grown on different types of media. Cuttings were grown on the combined media of 25% rice husk ash + 25% coco peat + 50% vermicompost obtained the highest number of roots (18.27 roots/cutting) and higher than the control (14.67 roots/cutting). While, the mixture of 50% sand: 25% rice husk ash: 25% coco peat (14.67%) gave lest root number. This suggested that when sand was replaced with organic substances helped to enhance organic content, nutrients as well as improve the water holding capacity of the growing media. It turned out that a higher organic content media could support the cuttings to form more roots.

However, different types of media did not affect the root length of cuttings. At the time of 75 DAPs, the root length ranged from 18.03 to 23.07 cm, relatively equivalent to the deep of the media. The porosity of the different types of media ranged from 53,330 to 61,268% (Table 2). There was no significant difference in the ability of roots to penetrate inside the growing media.

3.2. Effect of growing media on shoot length, number of leave and chlorophyll content of *Cyclea barbata*

The leaves are the main harvested part of *Cyclea barbata*. Leaves are distributed on the shoot, so the shoot has a high length, which is the basis for forming a large number of leaves. Besides, the number and the chlorophyll content index of leaves indicate the photosynthetic capacity of the plant which relates to the harvest yield. When growing on a suitable media, the plant usually has a large number of leaves and a high chlorophyll content index.

The results of Table 4 show that the length of shoots and the number of leaves of the cuttings were significantly different under influence of dif-

ferent types of media at the time of 75 DAPs. The cuttings grew on a mixed media including 25% husk ash + 25% coco peat + 50% vermicompost reached the highest shoot length (116.41 cm) and higher than the control (83.05 cm). Cuttings planted cut a mixed media of 25% sand + 50% rice husk ash + 25% coco peat got the lowest shoot length (72.17 cm).

Similarly, cuttings were planted on a mixed media of 25% husk ash + 25% coco peat + 50% vermicompost gave the highest number of leaves (15.53 leaves/cutting), even this was not statistically different from other types of media. While the cuttings planted on mixed media of 50% rice husk ash + 50% coco peat got the lowest leaf number (8.33 leaves/cutting).

Thus, it was clear that addition of vermicompost to growing media helped plants grow better. It was also obvious that the shoot length and number of leaves of cuttings grew in media containing vermicompost thanks to an improvement in the content of essential macro as well as micronutrients to the plantlets. This result was in agreement with the conclusions by Bhadwaj (2014), Vo & Wang (2014) and Pham & Nguyen (2018) in the propagation of various plants. Leaves of the cutting planted on different types of growing media showed no statistically significant differences in leaf chlorophyll index (CCI). At the time of 75 DAPs, the leaf chlorophyll index of the cuttings ranged from 25.17 to 28.77 CCI.

3.3. Effect of growing media on fresh roots matter (g/cutting), dry roots matter, fresh shoot matter and dry shoot matter of *Cyclea barbata*

Biomass of shoots and leaves indicates the ability of the plant to absorb water and nutrients. Types of media have different physical and chemical properties effecting the biomass accumulation of cuttings.

The results of Table 5 show that the fresh and dry roots matter of cuttings was highest on mixed media of 25% rice husk ash + 25% coco peat + 50% vermicompost (4.08 and 0.54 g/cutting, respectively) and significantly different from the control. The cuttings planted on mixed media containing 25% sand + 25% rice husk ash + 50% coco peat gained the lowest fresh and dry root weight (1.60 and 0.21 g/cutting, respectively). The result indicated that different growing me-

Table 2. Physical and chemical properties of the media after 30 days of incubation

Parameter	Unit	G1	G2	G3	G4	G5	G6	G7	G8	G9
pH_{H_2O}(1:5)		6.492	6.411	6.214	6.134	6.690	6.296	6.493	6.215	6.285
EC (1:5)	mS/cm	1.703	2.224	2.336	2.557	3.372	3.496	3.484	2.776	3.052
Total N	%	0.113	0.115	0.223	0.225	0.699	0.913	0.806	0.669	0.605
Total organic	%	8.966	8.967	17.930	17.932	11.490	29.415	20.452	20.673	16.537
C/N ratio		58.713	57.667	60.575	59.746	16.440	28.218	25.377	30.901	27.334
Total P_2O_5	%	0.033	0.037	0.062	0.066	0.868	0.920	0.894	0.493	0.411
Total K_2O	%	0.090	0.091	0.178	0.178	0.769	0.943	0.856	0.560	0.610
Bulk density	g/cm^3	0.981	0.801	0.768	0.588	0.619	0.552	0.586	0.570	0.562
Density	g/cm^3	2.101	1.817	1.775	1.491	1.461	1.378	1.419	1.434	1.451
Porosity	%	53.330	55.923	56.770	60.586	57.634	59.920	58.743	60.266	61.268
Humidity	%	30.869	33.888	33.967	36.986	33.700	33.858	33.779	35.422	32.103

Table 3. Effect of growing media on number of roots and root length of *Cyclea barbata* 75 days after planting

Growing media	Parameters	
	Number of roots (root/cutting)	Length of root (cm)
50% S : 25% HA : 25% CP (Control)	14.67^{bcd}	21.63
25% S : 50% HA : 25% CP	14.00^{cd}	19.70
25% S : 25% HA : 50% CP	12.80^{d}	18.03
50% CP : 50% HA	16.83^{ab}	19.93
50% HA : 50% VC	16.43^{abc}	22.37
50% CP : 50% VC	16.33^{abc}	18.87
25% HA : 25% CP : 50% VC	18.27^{a}	23.07
25% HA : 50% CP : 25% VC	17.23^{ab}	22.57
50% HA : 25% CP : 25% VC	16.00^{abc}	22.07
CV (%)	9.03	11.23
F_{value}	4.30^{**}	1.78^{ns}

S: Sand, HA: Husk ash, CP: Coco peat, PT: vermicompost; In the same column, numbers with the same character are statistically insignificant difference; ns: non-significant, **: the difference is statistically significant at $P = 0.01$.

Table 4. Effect of growing media on shoot length, number of leave and Content of Chlorophyll Index of *Cyclea barbata* 75 days after planting

Growing media	Parameters		
	Shoot length (cm)	Number of leave (leave/cutting)	Content of Chlorophyll Index (CCI)
50% S : 25% HA : 25% CP (Control)	83.05^{bc}	11.43^{bc}	25.17
25% S : 50% HA : 25% CP	79.93^{bc}	11.27^{bc}	28.77
25% S : 25% HA : 50% CP	72.17^{c}	9.33^{cd}	27.23
50% CP : 50% HA	80.13^{bc}	8.33^{d}	28.67
50% HA : 50% VC	101.85^{ab}	13.60^{ab}	28.53
50% CP : 50% VC	98.97^{ab}	13.90^{ab}	25.37
25% HA : 25% CP : 50% VC	116.41^{a}	15.53^{a}	25.73
25% HA : 50% CP : 25% VC	100.48^{ab}	14.70^{a}	27.17
50% HA : 25% CP : 25% VC	89.41^{bc}	13.73^{ab}	27.73
CV (%)	12.50	12.34	9.22
Fvalue	4.54^{**}	7.77^{**}	0.97^{ns}

S: Sand, HA: Husk ash, CP: Coco peat, PT: vermicompost; In the same column, numbers with the same character are statistically insignificant difference; ns: non-significant, **: the difference was statistically significant at $P = 0.01$.

dia did not impact statistically significant to both fresh and dry weights of the cuttings.

Similarly, fresh and dry leaf weights differed significantly when the cuttings were grown on different types of media. Fresh weight and dry were highest when cuttings were planted on a growing media containing 25% husk ash + 25% coco peat + 50% vermicompost (5.91 and 1.68 g/cutting, respectively) and higher than the control. However, this was not statistically different from weight of cuttings plated on mixed media of 25% rice husk ash + 50% coco peat + 25% vermicompost (5.45 and 1.50 g/cutting). It was clear that the media supplemented with vermicompost became lack of nutrients in the media is lower, thus affecting the growth of the cuttings. Cuttings planted on mixed media of 25% sand + 25% rice husk ash + 50% coco peat (3.26 and 0.79 g/tree, respectively) gained the lowest weight. These results were consistent with that ofreported by Vo & Wang (2014).

3.4. Effect of growing media on Dickson Quality Index, dry roots matter, fresh shoot matter and dry shoot matter of *Cyclea barbata*

The plantlet quality estimated based on Dickson Quality Index (DQI) is considered as a mea-

Table 5. Effect of growing media on fresh roots matter (g/cutting), dry roots matter, fresh shoot matter and dry shoot matter of *Cyclea barbata* 75 days after planting

Growing media	Parameters			
	Fresh roots matter (g/cutting)	Dry roots matter (g/cutting)	Fresh shoot matter (g/cutting)	Dry shoot matter (g/cutting)
50% S : 25% HA : 25% CP (Control)	2.08^{d}	0.25^{dc}	4.35^{bc}	1.10^{bcd}
25% S : 50% HA : 25% CP	1.88^{de}	0.23^{dc}	3.72^{cd}	0.90^{cd}
25% S : 25% HA : 50% CP	1.60^{e}	0.21^{d}	3.26^{d}	0.79^{d}
50% CP : 50% HA	2.87^{c}	0.32^{c}	3.48^{cd}	0.86^{d}
50% HA : 50% VC	3.31^{b}	0.43^{b}	5.05^{ab}	1.39^{ab}
50% CP : 50% VC	3.91^{a}	0.49^{ab}	5.09^{ab}	1.42^{ab}
25% HA : 25% CP : 50% VC	4.08^{a}	0.54^{a}	5.91^{a}	1.68^{a}
25% HA : 50% CP : 25% VC	3.71^{ab}	0.48^{ab}	5.45^{a}	1.50^{ab}
50% HA : 25% CP : 25% VC	3.34^{b}	0.43^{b}	4.88^{ab}	1.29^{ab}
CV (%)	7.88	13.88	12.55	19.13
F_{value}	46.38^{**}	16.94^{**}	7.75^{**}	5.58^{**}

S: Sand, HA: Husk ash, CP: Coco peat, PT: vermicompost; Values followed by different lowercase letters in superscripts were significantly different at $P < 0.01$.

Table 6. Effect of growing media on Dickson Quality Index, dry roots matter, fresh shoot matter and dry shoot matter of *Cyclea barbata* 75 days after planting

Growing media	Parameters		
	Dickson Quality Index	Percentage of live cuttings (%)	Percentage of Commer-cialarable cuttings (%)
50% S : 25% HA : 25% CP (Control)	0.016^{ab}	58.89	50.00
25% S : 50% HA : 25% CP	0.014^{b}	60.00	51.11
25% S : 25% HA : 50% CP	0.013^{b}	56.67	50.00
50% CP : 50% HA	0.014^{b}	60.00	52.22
50% HA : 50% VC	0.017^{ab}	62.22	54.45
50% CP : 50% VC	0.019^{a}	61.11	56.67
25% HA : 25% CP : 50% VC	0.019^{a}	64.44	57.78
25% HA : 50% CP : 25% VC	0.019^{a}	63.33	57.78
50% HA : 25% CP : 25% VC	0.019^{a}	61.11	54.44
CV (%)	13.662	6.23	9.46
Fvalue	3.680^{*}	1.14^{ns}	1.15^{ns}

S: Sand, HA: Husk ash, CP: Coco peat, PT: vermicompost; In the same column, numbers with the same character are statistically insignificant difference; ns: non-significant, *: the difference is statistically significant at $P = 0.05$.

sure for evaluating morphological characteristics (Johnson & Cline, 1991), which is a good indicator of seedling and plantlet quality based on the calculation of the healthiness and biomass distribution (Fonseca, 2002).

Table 6 shows that cuttings grown on different growing media differed statistically significantly ($P < 0.05$). In which, cuttings were grown on a mixed media containing vermicompost reached the best quality (0.019) and was not significantly different from the control (0.016). The mixed media of 25% sand + 25% rice husk ash + 50% coco peat showed the lowest plantlet quality (0.013).

Table 6 also shows that the survival percentage and ratio of Commercialarable plantlets achieved from different media were not statistically different. At the time 75 DAPs, the survival rate of cuttings ranged from 58.89 to 64.44% and the percentage of Commercialarable plantlets ranged from 50.11 to 57.78%.

Table 7. Financial efficiency for 1,000 cuttings

Growing media	Total revenue (VND/1000 cuttings)	Total expenditure (VND/1000 cuttings)	Profit (VND/1000 cuttings)	Profit ratio (time)
50% S : 25% HA : 25% CP (Control)	7,500,000	4,526,200	2,973,800	0,66
25% S : 50% HA : 25% CP	7,666,500	4,526,200	3,140,300	0,69
25% S : 25% HA : 50% CP	7,500,000	4,601,200	2,898,800	0,63
50% CP : 50% HA	7,833,000	4,601,200	3,231,800	0,70
50% HA : 50% VC	8,166,000	5,251,200	2,914,800	0,56
50% CP : 50% VC	8,500,500	5,401,200	3,099,300	0,57
25% HA : 25% CP : 50% VC	8,667,000	5,326,200	3,340,800	0,63
25% HA : 50% CP : 25% VC	8,667,000	5,001,200	3,665,800	0,73
50% HA : 25% CP : 25% VC	8,166,000	4,926,200	3,239,800	0,66

S: Sand, HA: Husk ash, CP: Coco peat, PT: vermicompost.

3.5. Financial efficiency for 1,000 cuttings of *Cyclea barbata*

Financial efficiency is an important factor that is interested in producers. Finding a suitable type of media can help reduce expenditure and increase revenue to achieve high profits.

The financial efficiency of Cyclea barbata cuttings on different types of media is presented in Table 7. The results showed that the plantlets planted on mixed media of 25% husk ash + 50% coco peat + 25% vermicompost achieved the highest profit (3,665,800 VND/1,000 plantlets) with a profit margin of 0.73 times. The plantlets planted on mixed media of 25% sand + 25% rice husk ash + 50% coco peat got the lowest profit (only 2,898,800 VND/1,000 plantlets).

The growing media 25% husk ash : 25% coco peat : 50% vermicompost gave the best growth and seedling quality, but not statistically different from the 25% husk ash growing media: 50% coco peat: 25% vermicompost. On the other hand, the addition of large amounts of vermicompost (50%) to the media increases investment costs, thereby reducing profits and profit margins.

4. Conclusions

Cuttings of *Cyclea barbata* planted the mixed media of 25% husk ash + 50% coco peat + 25% vermicompost was the best among tested media, the plantlet planted on media got highest number of roots (17.23 roots/cutting), longest shoot length (100.48 cm), highest number of leaves (14.70 leaves/cutting) as well as fresh and dry root matter (3.71 and 0.48 g/cutting), fresh and dry shoot matter (5.45 and 1.50 g/cutting). DQI coefficient reached the highest (0.019) at 75 DAPs, the profit was 3,665,800 VND/1000 cuttings with a profit margin of 0.73 times.

Conflict of interest

The authors declare no conflict of interest.

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Morphological and molecular characterization of plant growth promoting salt-tolerant bacteria associated with halophytes in the Southeast seaside of Vietnam

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ABSTRACT

Halophytes are found in high-salt environments naturally, and their roots may be associated with promising microbial candidates for promoting crop growth and salt tolerance. In this study, halotolerant bacteria were isolated from soil and root samples of *Rhizophora apiculate* (*R. apiculate*), *Avicennia officinalis* (*A. officinalis*), *Thespesia populnea* (*T. populnea*), *Acanthus ilicifolius* (*A. ilicifolius*) and *Trichophorum cespitosum* (*T. cespitosum*), five native halophytes of southeast seaside of Vietnam. Isolates were tested for maximum salt tolerant and screened for the ability of phosphate solubilization and indole acetic acid (IAA) production. Colony morphology, pigmentation, and Gram staining of each IAA production halotolerant isolate were determined. The bacterial isolates showed the highest salt tolerance and IAA production were identified by sequencing the 16S rRNA gene. A total of 54 isolates which were able to grow in the presence of up to NaCl 3M were isolated. Twenty-three halotolerant bacterial isolates had the capacity of IAA production, 60.9% from which were Gram positive with a cocci shape, colony in opaque/transparent yellow or opaque/off white, 1 - 2 mm or 2 - 3 mm in diameter with the convex surface. Three isolates VTDD1, VTTD2, and KGOR1 were able to solubilize insoluble phosphorus. The highest IAA production was observed in VTDR1 (93.77 µg/mL) followed by VTMR1 (75.23 µg/mL) and VTDR2 (60.00 µg/mL), while the smallest IAA production was observed in CGOD1 (0.50 µg/mL). The isolates VTDR1 and VTDR2 were identified as *Salinicola tamaricis* (99.58% and 99.67% identity respectively), while VTMR1 was found to be *Salinicola peritrichatus* (98.37% identity).

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1. Introduction

Soil salinization is a significant problem that affects and degrades land. It is widespread in desert or semi-arid places and has a direct impact on the sustainability of agricultural cultivation around the world, even posing a threat to global food security (Aragüés et al., 2015). It is one of the agricultural sector's most pressing problems because of its direct impact on the productivity and quality of agricultural products (Wild, 2003). Various approaches have recently been used to solve the problem of soil salinity, one of which is using plant growth-promoting rhizobacteria (PGPR) associated with halophytes.

Halophytes are naturally salt-tolerant plants that can stand up to concentrations of 1M NaCl (Kumari et al., 2015). Halophytic plants have

evolved various strategies to live in different salinity levels, one of which is ability to exploit the benefits provided by microbial systems around their roots, including endophytes and rhizosphere microorganisms (Sgroy et al., 2009; Ruppel et al., 2013).

There are many salt-tolerant halophyte rhizosphere and endophytic bacteria were investigated and reported to have the potential to contribute significantly to the ability of plants to adapt to adverse conditions (Numan et al., 2018). Salt tolerant bacteria that had the ability to grow at varied NaCl concentrations ranges from 50 to 600 mM (Zerrouk et al. 2019), from 1M to 4M (Kearl et al., 2019) or from 2% to 10% (Sharma et al. 2021). Among the salt-tolerant microorganisms associated with halophytes, PGPR has been effective at improving plant stress tolerance (Etesami & Beattie, 2018; Etesami & Maheshwari, 2018), especially phosphate solubilizing and IAA production bacteria. In the saline soil halotolerant bacteria reduce plant uptake of sodium ions from the soil by forming the biofilms in the rhizosphere that trap water and nutrients (Nadeem et al., 2014). Some other halophilic bacteria could stimulate plant growth based on binding of salt ions by the bacteria or production of volatile compounds or other signals that stimulate expression of genes to enhance growth via increased photosynthesis or other changes in the host plant (Meena & Meena., 2017; Numan et al., 2018). Phosphate (P) solubilizing bacteria convert unavailable P into available P, consequently improving the P supply available to the plant. Applying of phosphate solubilizing bacteria also decreases the pH of the soil and forms a P-offering microarea around the plant rhizosphere, and strengthening the activity of other beneficial microorganisms, promote the absorption of nutritive element ions of plant in salinity soil (Chen & Liu, 2019). In extremely condition plants are tried to use exogenous IAA hormone, that produced by rhizosphere organisms to improve budding and root hair formation. Root hair growth is stimulated by the presence of rhizosphere bacteria that produce IAA (Larekeng et al., 2020). These groups of bacteria were identified and used as inoculants to stimulate growth of non-host plants under saline conditions, and reduce pressure on arable lands. In the present study, bacteria were isolated from saline soils and roots of halophytes, and then screened for salt tolerance, phosphate solubilization, and IAA production.

2. Materials and Method

2.1. Materials

Total 30 soil and root tissues samples of halophytes *Rhizophora apiculate* (*R. apiculate*), *Avicennia officinalis* (*A. officinalis*), *Thespesia populnea* (*T. populnea*), *Acanthus ilicifolius* (*A. ilicifolius*) and *Trichophorum cespitosum* (*T. cespitosum*) and three soil samples of bare areas.

Chemicals: Yeast extract (India), meat extract (India), NaCl (99%, India), K_2HPO_4 (99.5%, China), L-triptophane ($\geq$ 99.0%, Germany), KCl (99%, Germany), Na_2HPO_4 ($\geq$ 99.0%, China), KH_2HPO_4 (99%, China), Tryptone (India), IAA (IAA $\geq$ 99.0%, China), $FeCl_3$.6H2O (98%, China), Agar powder (100%, Vietnam) $MnSO_4.H_2O$ (99%, China), $FeSO_4.7H_2O$ (99%, China) and $Ca_3(PO_4)_2$ (99%, China).

Equipment: Biosafety level 2AC2-6E8 incubator (2AC2-6E8, Esco, Singapore), MC40L autoclave (MC40L, ALP, Japan), Shaker HS260 (HS 260 control (0003066700), IKA, Germany), Spectrophotometry (NanoVue plus, Bichochrom, US), Freezer (MPR-414F-PE, Panasonic (PHC Corporation, Japan), single-channel micropipette 10÷1000 µL (Nichipet EXII, Japan), pH meter LAB845 (SI Analytics, Germany), ultrasound waves (S300H, Elma, Germany).

2.2. Collection of samples

Samples were collected as described by Kearl et al. (2019) from predominant halophyte species, such as *R. apiculate*, *A. officinalis*, *T. populnea*, *A. ilicifolius* and *T. cespitosum* around the seaside of Vung Tau (coordinate: 10°26'51.2"N 107°08'50.8"E), Can Gio (coordinate: 10°23'22.2"N 106°55'16.3"E) and Kien Giang (coordinate: 10º06'15.9"N 104°52'19.8"E). Five pooled soil and five pooled root tissue samples from each coordinate were collected. Each pooled sample was collected from 5 to 10 individual plants of each halophyte species. Samples were stored at 4ºC until be analyzed. Soil was also collected from bare areas where no plants were growing for comparison. Soil electrical conductivity (EC) as dS/m was measured using a Beckman RC-16C conductivity bridge. Soil samples were mixed with deionized water, the saturated mix was allowed to sit overnight for the soil to settle, and the pH of the liquid was measured with a standard pH meter.

2.3. Isolation and Characterization of salt tolerant Bacteria

Rhizosphere soil samples were left at room temperature for 24 h, then 0.5 g sample was vortexed in 1 mL sterile 1 X PBS buffer (phosphate buffered saline containing of NaCl 8.5 g/L, KCl 200 mg/L, Na_2HPO_4 1.44 g/L, KH_2PO_4 245 mg/L, pH 7.4) and plated on Luria broth (LB) agar plates containing of 1 M NaCl. To isolate endophytic bacteria, root samples were surface sterilized (by washing twice in sterile distilled water, once for 10 min in 70% ethanol, and twice in sterile 1 X PBS buffer) and ground in PBS buffer. Single colonies were continuously subcultured on Luria agar (LB) medium containing 1M NaCl to obtain pure colonies. Cultures were re-streaked on LB media containing increasing amounts of NaCl (2M, 3M, 4M) to determine the maximum salt tolerance of each isolate (Sharma et al., 2021). Colony morphology, pigmentation, and Gram staining of each isolate were also determined. Stock cultures of each isolate were stored at -20°C in 50% glycerol.

2.4. Phosphate solubilization

Phosphorus solubilizing activity of the isolates was determined qualitatively according to the method described by Nautiyal (1999) on Pikovskaya's agar medium. $Ca_3(PO_4)_2$ (0.5%) was used as the inorganic form of phosphate. The bacteria were spotted on the center of the plates, and the plates were then incubated at 28°C for 7 days. The experiment for each isolate were repeated for a total of three replicates. Transparent halo zone around the bacterial colony indicates the phosphate solubilizing activity of the bacterial isolates.

2.5. Characterization of indole acetic acid production

To determine IAA producing capability, and amounts of IAA produced by each salt tolerance isolate, a colorimetric technique was performed with Salkowski reagent using the Salkowski's method (Ehmann, 1977). The isolates were grown in L-triptophan Luria broth and incubated at 28°C for 4 days. The bacterial suspension was centrifuged at 4000 rpm for 20 min, then 2 mL supernatant was collected and mixed with 8 mL of Salkowski's reagent (2% $FeCl_3$ in 36% H_2SO_4 solution) to determine IAA producing capability. If the mixture color changed to pink, the mixture was kept in the dark for 30 min to determine the amount of IAA produced (Sarwar & Kremer, 1995) by recording the optical density (OD) at 530 nm using spectrophotometer. The experiment for each isolate were repeated for a total of three replicates. Sterile LB medium (with and without L-tryptophan) were used as controls.

2.6. Bacterial Identification

The bacterial isolates with high salt tolerance and IAA production were selected for identification. Genomic DNA was obtained from individual isolates using a DNA isolation kit GeneJET Plant Genomic DNA Purification - Thermo (America). The 16S ribosomal RNA (rRNA) gene was amplified by PCR with MyTaq™ DNA Polymerase - Bioline using the 63F and 1492R primers (Turner et al., 1999). The PCR products were submitted for sequencing at the Biotechnology Department of University of Science, Vietnam National University Ho Chi Minh City. The obtained sequences were were analyzed and determined by the BLAST program on the NCBI (https://www.ncbi.nlm.nih.gov).

3. Results

3.1. Isolation and characterization of rhizospheric and endophytic salt tolerance bacteria

Total 30 pooled soil and root tissue samples were collected around the dominant halophyte species at seaside of Vung Tau, Kien Giang, Can Gio areas including *R. apiculate*, *A. officinalis*, *T. populnea*, *A. ilicifolius* and *T. cespitosum* (Figure 1).

Soil salinity around the plants ranged from 4.10 to 12.75 dS/m (Table 1), according to Kotuby-Amacher et al. (2000) and Richards (1954), soils are moderately saline and strongly saline. In the bare-no plant, salinity was varied between 13.90 and 16.40 dS/m depending on the areas. The pH of all soil samples was ranged from 4.00 to 7.90 (Table 1).

Fifty-four isolates of bacteria were isolated from the rhizosphere soil and root samples on LB agar plates containing 1 M NaCl. Among which, 27 isolates were endophytic bacteria and 27 isolates were rhizosphere bacteria. The highest salt

Figure 1. Photos of the halophyte species: a) *R. apiculate*; b) *A. officinalis*; c) *T. populnea*; d) *T. cespitosum*; e) *A. ilicifolius*.

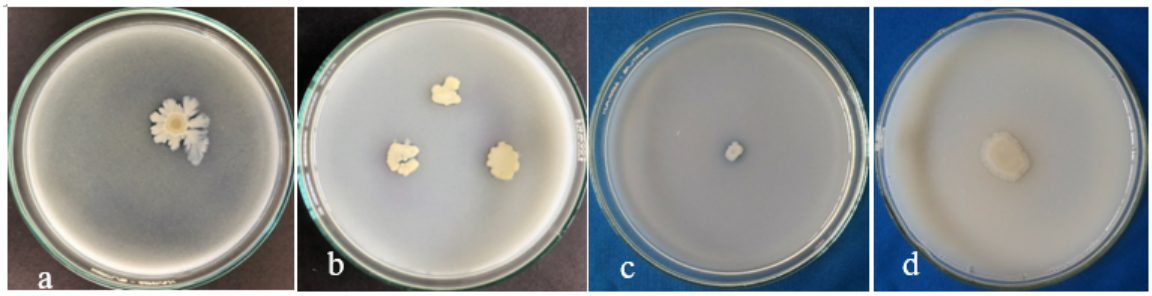

Figure 2. The phosphate solubilizing ability of three selected isolates a) VTDD1; b) VTTD2; c) KGOR1, and d) KGOD1 the isolate without solubilizing phosphate.

Table 1. Physicochemical analysis of soil samples

Plant species	Vung Tau		Can Gio		Kien Giang	
	EC dS/m	pH	EC dS/m	pH	EC dS/m	pH
Rhizophora apiculate	6.27	5.00	7.27	5.50	11.56	7.00
Avicennia officinalis	4.10	4.52	5.10	5.12	6.70	7.50
Thespesia populnea	8.50	5.00	9.62	5.83	4.90	5.45
Trichophorum cespitosum	4.50	4.00	4.87	4.30	4.68	5.40
Acanthus ilicifolius	5.62	5.95	7.52	5.90	12.75	6.30
Bare-no plants	13.90	5.35	14.93	5.05	16.40	7.90

concentration tolerance for growth up to 3 M was discovered in a variety of isolates. There were 18 isolates (33.3%) growing in the presence of NaCl 3 M, 32 isolates (59.3%) growing in the presence of NaCl 2M, and 4 isolates (7.4%) growing in the presence of NaCl 1 M. High salt tolerant rhizospheric isolates were found in associated with *R. apiculate* and *A. ilicifolius*, while the high salt tolerant endophytic isolates was found in *T. cespitosum*. The isolates grew equally well on minimal media agar plates at the same salt concentrations. The Gram staining, cell morphology, pigmentation and colony morphology were recorded for some isolates (Table 2). In LB plates containing 1M NaCl, none of rhizosphere bacteria were observed from soil sample of bare-no plants.

Most of the isolates are Gram positive, cell of isolates had a cocci shape (60.9% of bacterial isolates), and colony in opaque/transparent yellow or opaque/off white, 1-2 mm or 2-3 mm in diameter and a convex surface. Some others were found to have rod or short rod shape of cell, sometimes with opaque white or yellowish white colony with flat surface, core in centre or coreless.

3.2. Characterization of salt-tolerant bacterial isolates for ability of phosphate solubilization

The method for determination of phosphate solubilizing ability is based on the formation of visible halo/zone on Pikovskaya agar plates. In the present study, the phosphate solubilization was investigated in three salt-tolerant bacterial isolates VTDD1, VTTD2 and KGOR1. However, the visible halo zones on agar plates created by these isolates were very weak (Figure 2).

Table 2. Morphological characteristic of Salt tolerant and indole acetic acid production bacteria

Rhizosphere bacterial isolates				
Bacterial isolate	Plant species	Gram staining and cell morphology/ Pigment and morphology of the colony	Salt concentration tolerance	IAA, µg/mL
VTTD2	*T. populnea*	Gram + cocci/ opaque yellow 1-2 mm in diameter, convex surface, core in centre	3M	18.33
VTTD3	*T. populnea*	Gram + rod/ transparent yellow 2-3 mm in diameter, convex surface, core in centre	2M	48.20
KGTR1	*T. populnea*	Gram + cocci/ opaque yellow 1-2 mm in diameter, convex surface, core in centre	2M	41.10
CGMD3	*A. officinalis*	Gram - cocci/ off white 2-3 mm in diameter, the surface is smooth, shiny, and wet, coreless	3M	49.10
KGMR2	*A. officinalis*	Gram + cocci/ opaque white 3-6 mm in diameter, irregular, flattened surface, coreless	2M	12.00
CGDD1	*R. apiculate*	Gram + cocci/ off white 2-3 mm in diameter, the surface is smooth, shiny, and wet, coreless	3M	21.80
KGDR1	*R. apiculate*	Gram + cocci/ opaque white 1–2 mm in diameter, flat surface, core in centre	3M	47.97
KGOR2	*A. ilicifolius*	Gram + short rod/ yellowish white 1-2 mm in diameter, convex surface, and circular division, coreless	3M	19.10
CGOD1	*A. ilicifolius*	Gram + cocci/ pale orange 2-3 mm in diameter, slimy and fluid with a core	3M	0.50
CGOD2	*A. ilicifolius*	Gram + cocci/ orange 1-2 mm in diameter, slimy and fluid with a core	2M	59.30
CGCD1	*T. cespitosum*	Gram - cocci/ orange 1-2 mm in diameter, smooth, glossy, and wet surface, coreless	2M	30.30
CGCD2	*T. cespitosum*	Gram + cocci/ opaque white 2-3 mm in diameter, smooth, glossy and wet surface, coreless	2M	18.40
Endophytic bacterial isolates				
VTMR2	*A. officinalis*	Gram - short rod/ pale yellow 0,5-1 mm in diameter, clarity, convex surface	3M	32.00
VTMR1	*A. officinalis*	Gram - cocci/ translucent yellow, 1-2 mm in diameter, convex surface, core in centre	2M	75.23

Table 2. Morphological characteristic of Salt tolerant and indole acetic acid production bacteria (continued)

Endophytic bacterial isolates				
CGMR1	*A. officinalis*	Gram + cocci/ pale yellow 1 - 2 mm in diameter, the surface is smooth, shiny, and wet, coreless	2M	37.10
VTDR1	*R. apiculate*	Gram + cocci/ transparent yellow 1 - 2 mm in diameter, convex surface, core in centre	2M	93.77
VTDR2	*R. apiculate*	Gram + short rod/ yellowish white 1 - 2 mm in diameter, convex surface, circular division, coreless	3M	60.00
KGDD1	*R. apiculate*	Gram + rod/ opaque white 1 - 2 mm in diameter, flat surface, core in centre	2M	15.50
KGMD1	*R. apiculate*	Gram + cocci/ opaque white 2 - 4 mm in diameter, convex surface, pink core	2M	19.77
KGCD1	*T. cespitosum*	Gram + rod/ transparent yellow 2 - 3 mm in diameter, convex surface, core in centre	3M	14.90
KGCD2	*T. cespitosum*	Gram + cocci/ opaque yellow 2 - 3 mm in diameter, coreless	3M	39.37
VTCR1	*T. cespitosum*	Gram + cocci/ opaque yellow 2 - 3 mm in diameter, coreless	3M	15.23
CGCR1	*T. cespitosum*	Gram + cocci/ off white 2 - 3 mm in diameter, dry and rough surface, coreless	2M	16.70

3.3. Characterization of salt-tolerant bacterial isolates for ability of indole acetic acid production

The IAA production of the bacterial isolates was determined based on the change in color of the culture supernatant after the addition of the Salkowski reagent. Twenty-three bacterial isolates from fifty-four salt tolerant isolates were observed able to produce IAA (Figure 3). The color density was varied depending on the level of IAA produced by the tested isolates, as in Figure 3h and Figure 3g are bright-less pink, in Figure 3b - 3f are pink and brighter pink, in comparison to positive control (Figure 3a) containing 50 µg/mL IAA, and negative control (Figure 3i). The interaction between IAA, produced by bacterial isolates and Fe elements in Salkowski reagent forms a complex compound $[Fe_2(OH)_2(IA)_4]$, that makes the pink as result of positive reaction. The brighter pink color indicates that the higher content of IAA produced by bacterial isolate (Susilowati et al., 2018).

Indole acetic acid concentration values were obtained by preparing a standard curve from IAA solution by suspending IAA in water and mixed by ultrasound waves at a concentration of 1 mg/mL and diluting to a concentration of 100, 90, 80, 70, 60, 50, 40, 30, 20, 10 and 0 µg/mL. Spectrophotometric test results show the correlation between IAA standard solution (x) and absorbance (y) by obtaining a regression equation $Y = 0.0006x + 0.0272$ with correlation coefficient $R^2 = 0.9833$, which was met the requirement of IAA curve standard (Brick et al., 1991). The ability of IAA production of endophytic and rhizosphere bacterial isolates from halophyte plants is presented in Table 2. The highest IAA concentration is produced by the VTDR1 isolate at 93.77 µg/mL, followed by the VTMR1 isolate at 75.23 µg/mL, and the VTDR2 isolate at 60.00 µg/mL. The lowest IAA concentration is produced by the CGOD1 isolate at 0.50 µg/mL. IAA is a key hormone for various aspects of plant growth that can regulate many physiological processes, such as cell division, differentiation, and protein

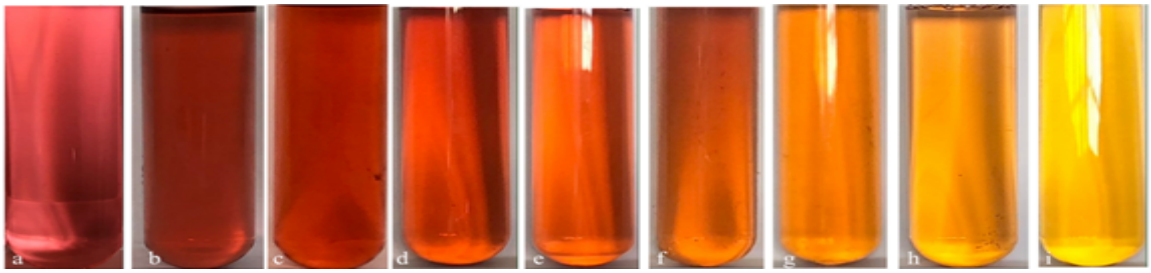

Figure 3. IAA test results of bacterial isolates based on differences in color obtained: a) IAA 50µg/mL; b)VTDR1; c)VTMR1; d)VTDR2; e)VTMR2; f)VTTD3; g)VTTD2; h)VTCR1; i) Salkowski.

synthesis. IAA produced by PGPR are required for improving root hair budding, formation, and growth, helping plants to cope with salt stress (Egamberdieva & Kucharova, 2009; Patil, 2011). IAA production varies greatly between species and strains, the host plant, different areas, and is also affected by environmental conditions, growth rates, and availability of substrates such as amino acids.

3.4. Bacterial identification

Three bacterial isolates (VTDR1, VTMR1 and VTDR2) with the highest IAA production and salt tolerance were selected for identification. The 16S rRNA gene from these isolates was successfully amplified. The PCR is about 1400 bp to 1500 bp (Figure 4) in length was sequenced. The 16S rRNA sequences of the bacterial isolates VTDR1 and VTDR2 show high identity (99.58% and 99.67%, respectively) to the *Salinicola tamaricis* sequence, whereas the VTMR1 show high identity (98.37%) in 16S rRNA sequence to *Salinicola peritrichatus*.

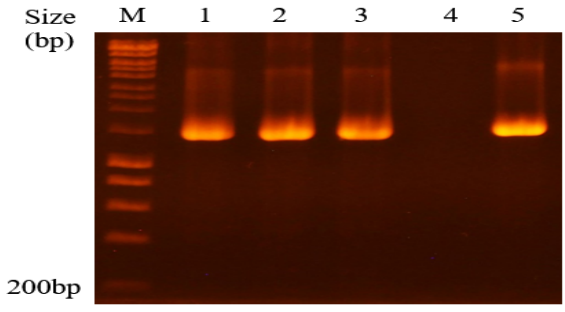

Figure 4. PCR amplified product of 16S rDNA of bacterial isolates. (Lad M: molecular size marker 1 kb; lane 1: isolate VTDR1; lane 2: VTDR2; lane 3: VTMR1; lane 4: control without DNA; lane5: DNA of *Bacillus subtilis*).

4. Discussion

Salinity stress which causes the decrease in yield of important crops as rice is increasing year by year in many countries due to global climate change. Every year, saline soil reduces agricultural cultivable area by 1 - 2 percent, leads to reducing food production. A strategy that has received much attention in recent years is using salt-tolerant microorganisms as plant growth-promoting rhizobacteria (PGPR) in combination with fertilizers to increase the salt tolerance of plants grown in flooded areas (Etesami & Beattie, 2018).

In the present study, the salt tolerant bacteria were isolated from saline soils and roots of the dominant halophytes in Southeast seaside of Vietnam as *Rhizophora apiculate*, *Avicennia officialist*, *Thespesia populnea*, *Acanthus ilicifolius* L., and *Trichophorum cespitosum*. A total of fifty-four isolates were found salt tolerant at different NaCl concentrations. About 33.3% of them have shown high salt tolerance at 3M NaCl. In a similar study, Kearl et al. (2019) isolated, screened, and characterized salt tolerant rhizosphere bacteria from the native halophyte of Ulta (*Salicornia rubra*, *Sarcocornia utahensis*, and *Allenrolfea occidentalis*). Forty-one isolates of three bacteria genus *Halomonas*, *Bacillus*, and *Kushneria* were demonstrated as salt tolerant bacteria. Some of which were able to grow in the presence of NaCl up to 4 M and stimulated plant growth of alfalfa in the presence of NaCl 1%. The diazotrophic salt tolerant bacterial strains of *Klebsiella*, *Agrobacterium*, *Pseudomonas*, and *Ochrobactrum* isolated from the roots of a halophytic plant (*Arthrocnemum indicum*) showed salinity tolerance ranging from 4% to 8%, and improved the productivity of peanut in saline conditions by increasing in total nitrogen (N) con-

tent up to 76% (Sharma et al., 2016). Zerrouk et al. (2019) found that *Pseudomonas plecoglossicida* strain Pp20 had the ability to grow at varied NaCl concentrations ranging from 50 to 600 mM. The study also revealed a positive impact on stem weight, seminal roots, lateral roots, and root length of maize in greenhouse experiment.

Different bacterial species stimulate the growth of a variety of plant species depending on the plant host and bacterial genus (Yuan et al., 2016). For example, the endophytic strain of *Bacilllus amyloliquefaciens* protects against salinity stress in rice by producing abscisic acid in response to increasing ability of glutamic acid and proline production of to increase resistance to salinity (Shahzad et al., 2017). In another study, Sarkar et al. (2018b) demonstrated that halotolerant *Enterobacter* sp. strain P53 can stimulate rice growth under salt stress due to the capacity of IAA production, HCN, siderophore and antioxidant activity. Mukhtar et al. (2020) found that, under salinity stress conditions halophilic PGP bacteria genus *Bacillus*, *Halobacillus*, and *Pseudomonas* associated with *Salsola stocksii* and *Atriplex Amnicola* improved plant growth and grain yield of maize by solubilization of phosphorus, nitrogen fixation and production of growth-promoting hormones indole acetic acid, siderophores and HCN.

In the present study, the bacterial isolates were further screened for the ability of phosphate solubilization and IAA production. There are only 3 bacterial isolates with the capacity to solubilize phosphate but the visible zones were weak. In the recent study, Sharma et al. (2021) reported that, the phosphate solubilization was demonstrated by three salt-tolerant bacterial isolates from saline agricultural fields of Haryana, India HB4N3, HB6P2 and HB6J2. However, the diameter of visible zones on the plates was impossible to measure, which was the same as the present study. We would suggest that, there are many factors that could affect the solubilization of insoluble phosphorus of halotolerant bacteria on Pikovskaya agar plates. Therefore, the method needs to be improved for optimization of the phosphorus solubilization capacity of salt tolerant bacteria.

There are twenty-three bacterial isolates in this study with capacity to produce IAA at different concentrations. The concentration of produced IAA was high in comparison with the others results. In previous results, IAA production of Bacillus sp. from saline agricultural fields of Haryana, India were ranges from 8.91 to 15.89 ppm (Sharma et al. 2021). *Bacillus subtillis* from cultivated soil sample in Egypt had capacity of IAA production with variable degrees ranged between 13.0-25.5 mg/L (Naeima, 2018). According to Mirza et al. (2001), IAA production by microorganisms depend on the culture conditions and substrate stage growth conditions and may vary between different species and strains of the same species. In present study, the endophytic bacteria could produce IAA at a higher concentration than rhizosphere bacteria from the same halophyte species. IAA synthesis by bacteria can be promoted by the presence of L-tryptophan (Mohite, 2013). L-tryptophan is an amino acid with an indole group that can function as a physiological precursor of IAA in plants and microorganisms because it contains active compounds that can enhance IAA biosynthesis, active microbial growth. IAA biosynthesis of microbes from plant roots, especially in the rhizosphere, which has a metabolism pathway through the synthesis of L-tryptophan derived from root exudates (Dewi et al., 2016).

The isolates VTDR1, VTMR1 and VTDR2 with high salt tolerance and IAA production ability were sequenced for amplified 16S rRNA gene and identified as Salinicola tamaricis (VTDR1, VTDR2) and Salinicola peritrichatus (VTMR1). In previous findings, many salt tolerance plant growth promoting rhizobacteria were identified as Klebsiella, Pseudomonas, Rhizobium, Azospirillum, Enterobacter, Serratia, Alcaligenes, Arthrobacter, Azotobacter, Burkholderia and Bacillus (Sarkar et al., 2018a; Ipek et al., 2019; Sayyed et al., 2019). Salinicola sp. might be a new bacterial species in PGPB group, that could be beneficial in improving crop yields in salt-affected agricultural fields.

5. Conclusions

Fifty-four isolates of rhizosphere and endophytic bacteria were able to grow in the presence of up to 3M NaCl were isolated from soil and root samples of five native halophytes of *Rhizophora apiculate*, *Avicennia officinalis*, *Thespesia populnea*, *Acanthus ilicifolius* and *Trichophorum cespitosum* in the southeast seaside of Vietnam. These bacterial isolates varied in pigmentation and colony morphology. Among which,

twenty-three halotolerant bacterial isolates produced IAA ranged from 0.50 µg/mL to 93.77 µg/mL, and three isolates had the ability of phosphate solubilization. The VTDR1 and VTDR2 isolates, which could grow in the presence of 3M NaCl, produced IAA at 93.77 µg/mL and 60.00 µg/mL, respectively, were identified as *Salinicola tamaricis* (99.58% and 99.67% identity, respectively). The VTMR1 isolate grew in 3M NaCl and produced IAA at 75.23 µg/mL was found to be *Salinicola peritrichatus* (98.37% identity).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Structural characteristics, tree species diversity and distribution of medicinal plant species at Ta Kou nature reserve, Binh Thuan province

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ABSTRACT

This article aimed to investigate the forest structural characteristics, tree species diversity for plant communities of the tropical moist evergreen close forest and distribution of medicinal plant species at Ta Kou nature reserve, Binh Thuan province. In this research, the structural characteristics and tree species diversity were analyzed from data collected from 20 typical plots with the size of 0.1 hectares. The location of medicinal plant species is identified by GPS through the route survey method. Research results show that IVi% index of dominant and co-dominant tree groups accounting for 26.4%. The number of trees is most concentrated in class $D_{1.3}$ < 20 cm and H < 10 m. The basal area and volume are mainly concentrated in class $D_{1.3}$ = 20 - 40 cm and H < 10 m. In the study area, 47 species of 27 plant families were found, in which the Dipterocarpaceae family has the most species; 28 medicinal plant species belong to 24 families, one of which is typical for the study area named "Thay Thim" tree (*Olax obtuse* Blume) and some other species are rare and threatened with extinction in Vietnam such as *Streptocaulon juventas* (Lour.) Merr., *Eurycoma longifolia* Jack, *Stemona collinsae* Craib, *Drynaria bonii* H. Christ.

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1. Introduction

Vietnam is located in the Indo-Burma region, which is one of 25 global biodiversity hotspots (Myers et al., 2000). The biodiversity of Vietnam ranks 16th in the world (WB, 2005). In terms of biogeography, Vietnam is the intersection of flora and fauna systems of India - Burma, South China and Indonesia - Malaysia. The above characteristics have made Vietnam a biodiversity center of the world with various natural ecosystems, rich in endemic species and genetic resources.

However, under the increasing pressure of poverty, livelihoods, prioritizing the development of the market economy, especially timber and wildlife products, special-use forests are often under pressure from many different causes. According to research results of the Institute of Forest Inventory and Planning, the main reason why Vietnam's natural forests have been declining significantly in recent years is due to the conversion of forest land use purposes, over-exploitation, especially in the Southeast, Central Coast and Central Highlands (PNR, 2017). The quality of forests and biodiversity in the special-use forest system across the country have been warned to be seriously declining, the extinction risk of some species is very high, including medicinal plant species.

Ta Kou nature reserve in Binh Thuan province is a national nature reserve with rich and diverse flora and fauna systems. It is noted that Ta Kou mountain alone (694 m altitude and 1104 ha) has about 159 medicinal plant species, accounting for 23% of the total number of plant species in this mountain. However, the coastal sandy soil

and dry climate that are typical for the region have made it difficult for agricultural production activities, causing the people's high dependence on forest resources. As a result, biodiversity and natural resources continue to decline (BTDARD, 2012).

Thus, the study on forest structure characteristics, tree species diversity and the distribution of medicinal plant species in Ta Kou nature reserve are of great significance, helping foresters identify the forest status quo such as tree species composition, density, stratum structure, forest volume, distribution of medicinal plant species, identify medicinal plant species used as medicinal plants and determine the use of each species, etc. Thus, there are development orientations for forest protection, nurturing and restoration, biodiversity, medicinal plant sources and proposals for specific conservation measures for sustainable forest development.

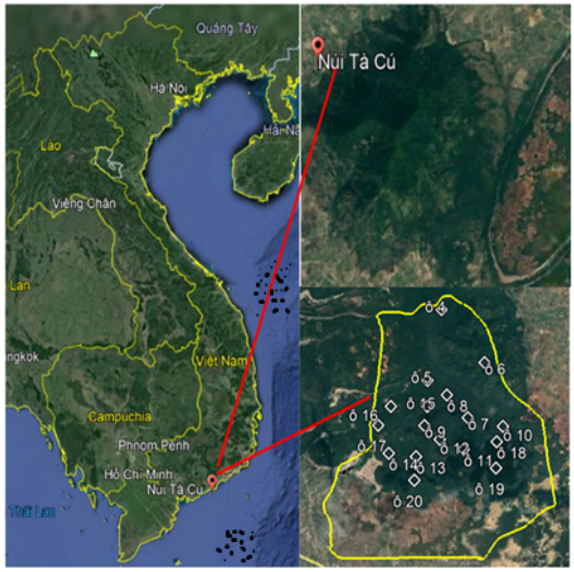

Figure 1. Location of sample plots in the study area.

2. Materials and Methods

2.1. Research subjects

The object of the study is the plant communities of the tropical moist evergreen close forest. The study forest's status is medium forest (forest classification according to Circular No. 33/2018/TT – BNNPTNT of the Ministry of Agriculture and Rural Development) (MARD, 2018).

Research location: Ta Kou nature reserve, located in Ham Thuan Nam district, Binh Thuan province, with geographical coordinates: 10041'28" to 10053'01" North latitude; 107052'14" to 108001'34" East longitude (Figure 1).

The study period is from September 2019 to May 2021.

2.2. Research methods

2.2.1. Sampling methods

Data was collected from 20 typical sample plots with the size of 0.1 ha for the medium forest status in the study area. In each sample plot, trees with $D_{1.3} \geq 8$ cm were counted by species (S, species), diameter at breast height ($D_{1.3}$ cm) and tree height (H, m). The tree species composition was determined according to Pham (2003), Tran & Nguyen (2003) and Vo (2004). D1.3 was determined using a tape measure with an accuracy of 0.1 cm. H was determined using a Blume-Leiss hypsometer with an accuracy of 0.5 m. The survey method was using the traverse line going through the established standard plots to investigate the medicinal plant species. The location of the standard plots and the medicinal plant species were determined by global positioning system (GPS).

2.2.2. Data processing methods

The species composition structure was determined based on the important value index (IVi%) by formula (1) (Misra, 1968) through 3 criteria: (i) Relative density (Ni%) is the percentage ratio between the density of studied species of i and total density of all species; (ii) Relative basal area (Gi%) is the percentage ratio between the basal area of the studied species i and total basal area of all species; (iii) Relative frequency (Fi%) is the percentage ratio between the occurrence frequency of a studied species i and total frequency of occurrence of all species. Dominant and co-dominant species have IVi $\geq$ 5%. Identifying tree species composition (S, species), diameter ($D_{1.3}$ cm), height (H, m), basal area (G, m^2) and volume (M, m^3). The identified tree species diversity indexes include: Number of timber species and species richness index, evenness index and species diversity index. Of which, species rich-

ness was determined by the number of species (S) and Margalef's species richness index (d or dMargalef) (Margalef, 1968). The evenness index was determined based on Pielou index (J') (Pielou, 1975). Tree species diversity was determined based on the Shannon - Weiner diversity index (H') (Shannon & Wiener, 1963) and the Simpson dominance index (λ') (Simpson, 1949). The tree species diversity of the plant communities was determined based on the diversity index β - Whittaker (1972). The tree species diversity indexes were determined by formula (2) - (6); where S = total number of timber species encountered in all n sample plots; s = the average number of timber species encountered in a sample plot, $P_i = n_i/N$ (N is the total number of trees in the sample plot, and n_i is the number of trees of species i), Ln() = logarithm of base Neper.

$$IVi = (Ni\% + Gi\% + Fi\%)/3 \quad (1)$$

$$d_{Margalef} = (S\text{-}1)/Ln(N) \quad (2)$$

$$J' = H'/H'max, \text{ with } H'max = Ln(S) \quad (3)$$

$$H' = -\sum_{i=1}^{S} P_i * Ln(P_i) \quad (4)$$

$$\lambda' = \sum (n_i * (n_i - 1)/(N * (N - 1)) \quad (5)$$

$$\beta - Whittaker = S/s \quad (6)$$

Research on medicinal plant species: the study referred to scientific documents of the authors Vo (2000), Do (2004) to accurately determine the scientific names, common names, morphological characteristics and uses of medicinal plants. Identification of medicinal plants on the list of rare and precious medicinal plants was based on the Vietnamese Red List of medicinal plants (2006), Decree No.06/2019/ND-CP dated January 22, 2019, of the Government and the Red Book of Vietnam (2007). The frequency of occurrence of medicinal plant species was calculated by the formula (7); where Ni is the number of trees of the species collected on the survey routes; N is the total number of trees collected on the survey routes. Mapinfo software was used to build a distribution map of medicinal plant species encountered in the study area.

$$Fi = (Ni/N)*100 \quad (7)$$

3. Results and Discussion

3.1. Plant family and tree species structure

3.1.1. Plant family structure

The results of analyzing 20 plant communities on 0.1-ha plots of the tropical moist evergreen close forest in the medium forest status in Ta Kou area, Binh Thuan Province showed that:

Forty-seven species of timber plants belonging to 27 plant families have been identified, of which the family with the most species is the Dipterocarpaceae family with 5 species. Next is the Anacardiaceae family with 4 species. Families such as Annonaceae family and Caesalpinioideae subfamily have 3 species, the remaining families have 1 to 2 species. Dipterocarpaceae family is the family with the largest number of individuals (333 individuals).

3.1.2. Tree species structure

In the study area, 47 species of trees were encountered (Table 1), of which *Shorea roxburghii* Roxb is the species with the highest IV% (7.00%), followed by these 3 species: *Careya sphaerica* Roxb, *Dillenia ovata* Wall, *Diospyros eriantha* Champ ex Benth. This is a dominant and co-dominant species group, which contributes an average of 26.4% in terms of N, G and F. In addition, 43 other species also contribute greatly to the species diversity and sustainable development of the forest. Species composition formula: 0.7Sr + 0.694Cs + 0.664Do + 0.582De + 7.36Os. Where: Sr stands for Shorea roxburghii Roxb, Cs for Careya sphaerica Roxb, Do for Dillenia ovata Wall, De for Diospyros eriantha Champ ex Benth, and Os for the other species.

3.2. Stand structure characteristics

3.2.1. Structure in terms of density, basal area and volume by diameter group

Density (N, trees/ha), basal area (G, m^2/ha) and volume (M, m^3/ha) in 4 diameter groups $D_{1.3}$ (< 20, 20 – 40, 40 - 60 and > 60 cm) are presented in Table 2.

Calculation results show that the average density is 1.067 trees/ha; in which the majority (60.3% or 643 trees/ha) is in the $D_{1.3}$ < 20 cm, the lowest is in the $D_{1.3}$ > 60 cm (0.2%

Table 2. Density, basal area and volume by diameter group in medium forest status

Group $D_{1.3}$ (cm)	N (number of trees)	G (m^2)	M (m^3)	Ratio (%)			
				N	G	M	Average
< 20	643	9.87	33.23	60.3	28.6	22.7	37.2
	159[1]	2.47	8.39	14.9	7.2	5.7	9.3
20 - 40	409	21.77	94.93	38.3	63.0	65.0	55.4
	148	8.35	37.34	13.9	24.2	25.6	21.2
40 - 60	13	2.31	14.02	1.2	6.7	9.6	5.8
	9	1.55	9.49	0.8	4.5	6.5	3.9
> 60	2	0.61	3.94	0.2	1.8	2.7	1.6
	1	0.24	1.45	0.1	0.7	1.0	0.6
Total	1.067	34,56	146.11	100.0	100.0	100.0	100.0
	317	12.61	56.67	29.7	36.5	38.8	35.0

Unit: 1 ha.
[1]The values in the bottom rows belong to dominant and co-dominant species or ecologically significant species groups.

Table 1. Species composition ratio (IV%) in high tree layer in medium forest status

No.	Species	N (number of trees)	G (m^2)	F (frequency)	Ratio (%)			
					N%	G%	F%	IVI%
1	*Shorea roxburghii* Roxb	80	3.62	85	7.50	10.47	3.03	7.00
2	*Careya sphaerica* Roxb	77	3.72	80	7.22	10.75	2.85	6.94
3	Dillenia ovata Wall	87	2.84	100	8.15	8.21	3.57	6.64
4	*Diospyros eriantha* Champ ex Benth	73	2.43	100	6.84	7.04	3.57	5.82
	Sum of 4 species	317	12.61	365	29.71	36.48	13.01	26.40
	Other species	750	21.95	2440	70.29	63.52	86.99	73.60
	Total	1.067	34.56	2805	100.0	100.0	100.0	100.0

Unit: 1 ha.

or 2 trees/ha). The basal area is 34.56 m^2/ha; in which the largest is in the group $D_{1.3}$ = 20 - 40 cm (63.0% or 21.77 m^2/ha), the smallest is in the group $D_{1.3}$ > 60 cm (1.8% or 0.61 m^2/ha). The total volume is 146.11 m^3/ha; in which the largest in is the group $D_{1.3}$ = 20 - 40 cm (65.0% or 94.93 m^3/ha), the smallest is in the group $D_{1.3}$ > 60 cm (2.7% or 3.94 m^3/ha). Dominant and co-dominant or ecologically significant species groups contribute N, G and M in all $D_{1.3}$ groups; in which the highest proportion is in the $D_{1.3}$ = 20 - 40 cm (21.2%) and the smallest percentage is in the $D_{1.3}$ > 60 cm (0.6%).

3.2.2. Structure in terms of density, basal area and volume by height class

Density (N, trees/ha), basal area (G, m^2/ha) and volume (M, m^3/ha) in three height classes (< 10, 10 - 15 và > 15 m) are presented in Table 3.

Calculation results show that the majority (84.1% or 897 trees/ha) is in the H class < 10 m, followed by class H = 10 - 15 m (15.6% or 166 trees/ha), the remaining 0.3% (4 trees/ha) is in class H > 15 m. The basal area is mainly in the class H < 10 m (66.5% or 22.99 m^2/ha), the smallest is in class H > 15 m (1% or 0.35 m^2/ha). Total volume is 146.11 m^3/ha; in which, the largest is in class H < 10 m (55.8% or 81.54 m^3/ha), the smallest is in class H > 15 m (1.7% or 2.54 m^3/ha). Dominant and co-dominant or ecologically significant species groups contribute N, G and M in all H classes; in which the highest proportion is in class H < 10 m (20.7%), followed by class H = 10 - 15 m (13.4%) and the smallest is in class H > 15 m (0.9%).

3.3. Plant family diversity and tree species diversity in the tropical moist evergreen close forest

3.3.1. Plant family diversity in the medium forest status

The results of analyzing 20 plant communities on 0.1-ha sample plots of the tropical moist evergreen close forest are presented in Table 4.

The average number of families encountered in the 0.1-ha sample plot was 20 families; ranging from 16 to 25 families, coefficient of variation CV = 12.2%. The average number of timber species ranges from 1 to 5 species/family. The

Table 3. Density, basal area and volume by height class in medium forest status

H class (m)	N (number of trees)	G (m^2)	M (m^3)	Ratio (%)			
				N	G	M	Average
< 10	897	22.99	81.54	84.1	66.5	55.8	68.8
	250[1]	7.19	26.06	23.4	20.8	17.8	20.7
10 - 15	166	11.22	62.03	15.6	32.5	42.5	30.2
	65	5.11	28.36	6.1	14.8	19.4	13.4
> 15	4	0.35	2.54	0.3	1.0	1.7	1.0
	2	0.31	2.25	0.2	0.9	1.5	0.9
Total	1.067	34.56	146.11	100.0	100.0	100.0	100.0
	317	12.61	56.67	29.7	36.5	38.8	35.0

Unit: 1 ha.
[1]The values in the bottom rows belong to dominant and co-dominant species or ecologically significant species groups.

families with the largest number of species are Dipterocarpaceae (5 species) and Anacardiaceae (4 species). The average density is 107 trees/0.1 ha; in which the Dipterocarpaceae family has the highest average number of individuals (17 trees/0.1 ha or 15.9%). The average abundance (d) of the families is 4.09 ranging from 3.24 to 5.09 CV = 12.3%. The average evenness of the families is 0.91/0.1 ha; ranging from 0.85 to 0.96 CV = 3.5%. The average diversity index H' is 2.72 the lowest is 2.44 and the highest is 2.97 CV = 6.0%. The average Simpson dominance index is 0.07 the lowest is 0.05. and the highest is 0.12. The sampling plots receiving the Simpson dominance index is 40% larger than the average value compared to the total number of sampling plots. In general, the plant family diversity of the natural medium evergreen broad-leaved mountain timber forest in study is only above the average level.

Comparing this result with that of Phan Minh Xuan (2019)'s research, the average number of families encountered in the sample plot herein is similar to that in Binh Chau - Phuoc Buu (BCPB) area which is 20 families and the families with the largest number of species are mainly Dipterocarpaceae and Anacardiaceae. However, the variation in the number of families among plant communities in the BCPB area is larger than Ta Kou area ($CV_{BCPB} = 16.3\% > CV_{Ta\ Kou} = 12.2\%$). Diversity indices (d, J', H') in Ta Kou area are respectively higher than BCPB area (Tcalculating (d) = 5.34; Tcalculating (J') = 4.86; Tcalculating (H') = 4.23; $P < 0.05$), showing that the diversity of plant families in Ta Kou area is higher than BCPB area.

Table 4. Statistical characteristics of plant family diversity for medium forest status

No.	Descriptive statistics	F (number of families)	N (number of trees)	d	J'	H'	λ'
1	Number of sample plots	20	20	20	20	20	20
2	Average	20	107	4.09	0.91	2.72	0.07
3	Standard Error	0.55	2.59	0.11	0.01	0.04	0.00
4	Range	9	37	1.84	0.11	0.53	0.07
5	Minimum	16	88	3.24	0.85	2.44	0.05
6	Maximum	25	125	5.09	0.96	2.97	0.12
7	Coefficient of variation (CV%)	12.2	10.8	12.3	3.5	6.0	26.4

Unit: 0.1 ha.

3.3.2. Diversity of tree species in medium forest status

The total number of timber plant species encountered in 20 plant communities on 0.1-ha plots is 47 species. The average number of timber plant species encountered is 28 species/0.1 ha; ranging from 22 to 35 species; CV = 12.6%. The average number of individuals is 107 trees/0.1 ha; ranging from 88 to 125 trees; CV% = 10.8% (Table 5).

The average species abundance index (d) is 5.80; ranging from 4.59 to 7.15. The abundance of timber plant species is quite even (J' = 0.92), ranging from 0.88 to 0.95. The average Shannon diversity index (H') is 3.08; ranging from 2.82 to 3.26. The average Simpson dominance index λ'

Table 5. Statistical characteristics of tree species diversity for the medium forest status

No.	Descriptive statistics	S (number of species)	N (number of trees)	d	J'	H'	λ'	β
1	Number of sample plots	20	20	20	20	20	20	20
2	Average	28	107	5.80	0.92	3.08	0.05	1.70
3	Standard Error	0.79	2.59	0.16	0.004	0.03	0.002	0.05
4	Range	13	37	2.56	0.07	0.44	0.03	0.79
5	Minimum	22	88	4.59	0.88	2.82	0.04	1.34
6	Maximum	35	125	7.15	0.95	3.26	0.07	2.14
7	Coefficient of variation (CV%)	12.6	10.8	12.3	2.0	4.6	18.4	12.8

Unit: 0.1 ha.

is 0.05; ranging from 0.04 to 0.07. The average β – Whittaker diversity index is 1.70; ranging from 1.34 to 2.14; the diverse components have relatively low variation; in which the one with the strongest variation is the Simpson dominance index λ' (CV = 18.4%); followed by the β - Whittaker index (CV = 12.8%) and the number of species (CV = 12.6%). The Pielou's evenness index (J') has the lowest variation among plant communities (CV = 2.0%).

A comparison between this result and that in the research of Nguyen (2018) and Phan (2019) shows common characteristics in all 3 study areas (Ta Kou, BCPB and Nui Ong): tree species are not evenly distributed on the standard plots, there is no statistically significant difference in the average number of tree species encountered in the sample plots (0.1 – 0.2 ha) with a range from 23 species (Nui Ong) to 29 species (BCPB) compared to 28 species in Ta Kou ($P > 0.05$). Coefficient of variation in number of species in Ta Kou area (CV = 12.6%) is lower than BCPB area (CV = 22.7%) and Nui Ong area (CV = 22.4%). The comparison results show that: J'and H' indices in Ta Kou area are statistically significantly higher than in BCPB area, respectively ($T_{\text{calculating (J')}} = 4.02$; $T_{\text{calculating (H')}} = 2.66$; $P < 0.05$); however, there is no statistically significant difference in species abundance index ($T_{\text{calculating (d)}} = 0.89$; $P > 0.05$). Meanwhile, d, J', H' indices in Ta Kou area are statistically significantly higher than in Nui Ong area, respectively ($T_{\text{calculating (d)}} = 3.43$; Tcalculating (J') = 2.99; $T_{\text{calculating (H')}} = 4.86$; $P < 0.05$). In general, the diversity of tree species in Ta Kou area is higher than in BCPB and Nui Ong areas. On the other hand, the diversity index β - Whittaker in the medium forest of Ta Kou ($\beta = 1.70$) is lower than that of Nui Ong ($\beta = 1.78$) and BCPB ($\beta = 3.76$). This proves that the tree species composition in Ta Kou area is more even than the other two areas. In other words, the environmental conditions under the medium forest canopy in the study area (Ta Kou) are more stable than the medium forest conditions in BCPB and Nui Ong areas.

3.4. Diversity of tree species by stand structure

The diversity of tree species based on four diameter groups $D_{1.3}$ (< 20, 20 – 40, 40 - 60 and > 60 cm) and three height classes H (< 10, 10 - 15 and > 15 m) of the medium forest in the study

area are presented in Table 6.

The analysis results show that the two factors S (number of species) and N (stand density) decrease gradually, following the increase of group $D_{1.3}$ and H class. The decrease of S and N leads to a decrease in the H' index and an increase in the λ' index. β – Whittaker index in the $D_{1.3}$ groups and H classes is the ratio between the total number of tree species encountered in 20 plant communities and the number of tree species encountered in each group D1.3 and class H. β – Whittaker index's value is 1 in group $D_{1.3} < 20$ cm and $D_{1.3} = 20 - 40$ cm; and class H < 10 m, indicating that most of the tree species are present in group $D_{1.3} < 20$ cm; $D_{1.3} = 20 - 40$ cm and class H < 10 m. In contrast, β - Whittaker index receives a high value in group $D_{1.3} >$ 60 cm and class H > 15 m, indicating that only a very small number of tree species reaches the largest size in the forest stand.

3.5. Distribution of medicinal plant species in the study area

3.5.1. List of medicinal plant species on 3 survey routes in the study area

The survey results on 3 routes showed that all 538 individuals used for medicinal purpose were recorded with different uses, belonging to 28 species and 24 families (Table 7). The families with the highest number of species are Fabaceae with 3 species accounting for 10.71%, Smilacaceae and Asteraceae with 2 species (accounting for 7.14%), the remaining 21 families has 1 species (accounting for 75%) (Figure 2).

Among the 28 species belonging to 24 investigated families, there is one typical species for the study area, which is "Thay Thim" tree belonging to the Olacaceae family. In addition, there are some species on the Red List of Vietnamese medicinal plants (2006) which are rare and threatened with extinction in Vietnam such as: *Streptocaulon juventas* (Lour.) Merr., *Eurycoma longifolia* Jack, *Stemona collinsae* Craib, *Drynaria bonii* H. Christ.) (Nguyen, 2016). The difference in occurrence frequency between the highest frequency species and the smallest frequency species is 15.1%.

Table 6. Diversity of tree species by diameter group and height class in medium–forest status

Structure	Group/class	S (number of species)	N (number of trees)	d	J'	H'	λ'	β
Diameter $D_{1.3}$ (cm)	< 20	47	643	6.43	0.94	3.61	0.03	1.00
	20 - 40	47	409	6.86	0.88	3.38	0.05	1.00
	40 - 60	10	13	2.76	0.90	2.06	0.12	4.70
	> 60	2	2	0.91	0.92	0.64	0.33	23.50
Height (m)	< 10	47	897	6.14	0.93	3.58	0.03	1.00
	10 - 15	46	166	7.76	0.86	3.28	0.05	1.02
	> 15	4	4	1.54	0.98	1.35	0.14	11.75

Table 7. List of medicinal plant species on 3 survey routes in the study area

No.	Scientific species	Scientific family
1	*Stemona collinsae* Craib.	Stemonaceae
2	*Abrus precatorius* L.	Fabaceae
3	*Tetracera indica* (L.) Merr	Dilleniaceae
4	*Gnetum montanum* Markgr	Gnetaceae
5	*Cyclea barbata* Miers	Menisperimaceae
6	*Pandanus odoratissimus* L.f.	Pandanaceae
7	*Mucuna pruriens* (L.) DC.	Fabaceae
8	*Caryota mitis* Lour.	Arecaceae
9	*Adiantum caudatum* L.	Adiantaceae
10	*Uraria crinita* (L.) Desv. ex DC.	Fabaceae
11	*Streptocaulon juventas* (Lour.) Merr.	Asclepiadaceae
12	*Smilax cambodiana* Gagnep.	Smilacaceae
13	*Smilax ovalifolia* Roxb.	Smilacaceae
14	*Dioscorea persimilis* Prain et Burk	Dioscoreaceae
15	*Passiflora foetida* L.	Passifloraceae
16	*Strychnos nux-vomica* L.	Loganiaceae
17	*Eurycoma longifolia* Jack	Simaroubaceae
18	*Phyllanthus emblica* L.	Euphorbiaceae
19	*Costus speciosus* Sm.	Costaceae
20	*Melastoma candidum* D. Don	Melastomaceae
21	*Nepenthes mirabilis* (Lour.) Druce	Nepenthaceae
22	*Eupatorium odoratum* L.	Asteraceae
23	*Emilia sonchifolia* DC.	Asteraceae
24	*Alpinia conchigera* Griff.	Zingiberaceae
25	*Polia arenaria* Lour.	Caryophyllaceae
26	*Rhodomyrtus tomentosa* (Ait.) Hask	Myrtaceae
27	*Drynaria bonii* H. Christ.	Polypodiaceae
28	*Olax obtuse* Blume	Olacaceae

3.5.2. Application of Google Earth in the management of medicinal plant species

From the information collection (coordinates, species name identification, morphological characteristics, uses, images...) of medicinal plant species discovered on 3 survey routes. Using Google Earth software, species description and their representation on the map application (Figure 3) can facilitate the monitoring and management of medicinal plant species. In particular, at the coordinates of a medicinal plant species there will be a set of information about its attributes to be noticed such as: species name, scientific name, scientific family, coordinates, morphology, uses (Figure 4)... This will help forest management agencies better manage the database collected from the field without having to go through notebooks or Microsoft editing software such as Word, Excel.

The survey results showed that, on the 3 survey routes, the medicinal plant species appeared around the study area are quite rich and diverse. This is very important information to help authorities and forest owners manage, monitor and check the medicinal plant species distribution status, locations of medicinal genetic resources with high scientific and application value, serving the development, replication and conservation purposes.

4. Conclusions

The study has identified 47 timber plant species belonging to 27 plant families, in which there are 4 dominant and co-dominant tree species accounting for 26.4%, including *Shorea roxburghii* Roxb, *Careya sphaerica* Roxb, *Dillenia ovata* Wall, *Diospyros eriantha* Champ ex Benth. The highest number of trees is in the group $D_{1.3} < 20$ cm and class H < 10 m, the smallest number is in group $D_{1.3} > 60$ cm and

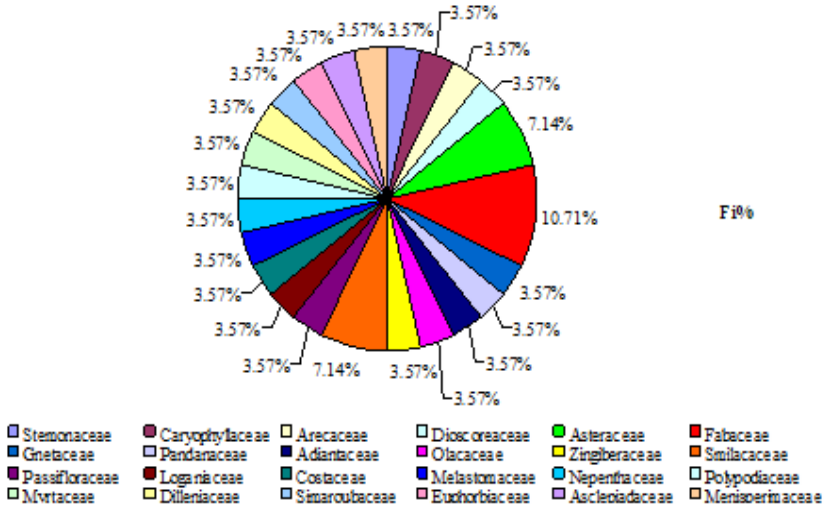

Figure 2. The species's frequency of occurrence on 3 survey routes.

Figure 3. Coordinates of medicinal plant species in the study area.

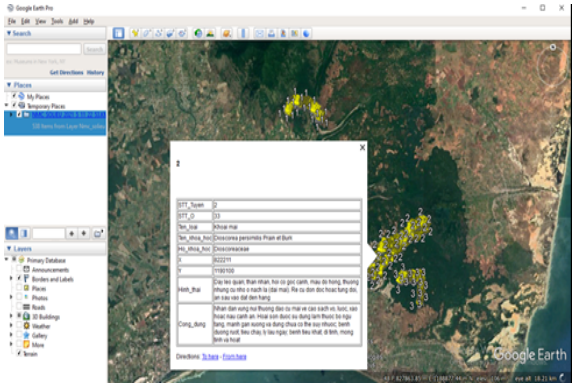

Figure 4. Managing medicinal plant species on Google Earth application.

class H > 15 m. The largest cross-section and timber volume are mainly in group $D_{1.3}$ = 20 - 40 cm and class H < 10 m, the smallest numbers are in group $D_{1.3}$ > 60 cm and class H > 15 m.

Analysis on plant family diversity and tree species diversity based on basic biodiversity indices such as Shannon – Weiner (H'), Pielou (J'), Margalef (d), Simpson (λ') indicated that the plant family diversity and tree species diversity in the study area is above average level.

The composition of medicinal plant species in the survey area is very rich, showing a great potential in medicinal species conservation and development. The use of Google Earth software helps the search, monitoring and management of medicinal plant species quick, convenient, and scientific.

Conflict of interest

The authors declare no conflict of interest.

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Evaluating the diversity of native ornamental fishes in Dong Nai biosphere reserve, Dong Nai province, Vietnam

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ABSTRACT

This study was conducted from May 2019 to December 2020 to investigate the diversity of native ornamental fishes in Dong Nai Biosphere Reserve (DNBR). The study identified 116 fish species belonging to 11 orders and 28 families in the water bodies of the DNBR. The analysis of compilation of the samples in the study further revealed that Cyprinidae was the most abundant family contributing 50 species followed by Perciformes and Siluriformes providing 24 species and 21 species, respectively. Out of 116 recorded species, 53 species were considered as ornamental fish, 77 species as food fish and 31 species as both ornamental and food fish. Among the ornamental fish group, many species have recorded good abundance at studied sites. Most of these fish species are high demand and sold at high prices in the domestic and international markets. The domesticated results showed that 21 ornamental fish species belong to 11 families were adapted and developed well in captivity conditions. The results also specified that eight species reach level 4 of domestication indicating truly domesticated, while the remaining 13 species belong to the first three levels of domestication, implying need to be further domesticated before being marketed. In addition, the study concluded that if managed sustainably, the collection of wild fish for the ornamental fish purpose could provide a stable income and livelihood for communities in the DNBR.

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1. Introduction

Aquarium keeping is one of the most popular hobbies with millions of enthusiasts worldwide today. It is also a good way to relax and reduce stress from work. The growing interest in aquarium species has resulted in steady increase in global aquarium trade. The international aquarium trade has grown dramatically over the past century, especially since the early 1980s, becoming one of the most popular amusements in global (Livengood & Chapman, 2011; Strecker et al., 2011). Today, millions of families around the world have owned at least one aquarium, especially in developed countries such as France, Italia, Germany, the United State of America, Japan (Rhyne et al., 2012; Papavlasopoulou et al., 2014)... Surveys of the world aquarium market showed that about 6,000 species of ornamental fish are traded annually, of which freshwater aquarium fish contribute approximately 4,000 species, accounting for about 75% of the total number of marketed species (Rhyne et al., 2012, 2012; Raghavan et al., 2013). It is estimated that close to 30-35 species of freshwater aquarium fish have dominated the global trade such as *Poecilia reticulata, Paracheirodon innesi, Xiphophorus maculatus, Xiphophorus helleri, Poecilia velifera, Pterophyllum scalare, Carassius auratus, Brachydanio rerio, Symphysodon* spp (Monticini, 2010)...

Historically, all ornamental fish sold on the

aquarium market were caught from the wild. Wild fish keeping as aquarium fish is becoming popular in many places in the world. With the high demand and prices of many beautiful species, native aquarium fish are being harvested from the wild in larger volumes and at higher rates by manual fishing methods using small seines, dip nets, and a variety of small trap nets (Vagelli & Erdmann, 2002; Cato & Brown, 2003; Lunn & Moreau, 2004). In addition, many reports indicated that catching fish for ornamental purposes is usually in small quantities but often has a higher value than large catches of the same species but for local uses such as human food, bait for fishing, or food for fish and animals (Davenport, 2016; King, 2019).

Dong Nai Biosphere Reserve (DNBR) is well known for its high level of biodiversity and of global meaningful ecosystem. The flora of DNBR includes 2,812 species of vascular plants belonging to 192 families, 99 orders. While the fauna includes 110 species of mammals belonging to 31 families, 12 orders; 348 bird species; 134 reptile and amphibian species; 175 fish species and 1,243 insect species (DNDNRE, 2017). The fish fauna of DNBR maintains many rare and endangered fish species recorded in the Vietnam Red Book and IUCN's Red List such as *Scleropages formosus*; and high economic fish species included *Anguilla marmorata, Hemibagrus wyckioides, Oxyeleotris marmoratus*; and many other rare fish species such as: *Morulius chrysophekadion, Chitala ornata, Probarbus jullieni, Cyclocheilichthys enoplos* (Nguyen et al., 2009). In addition, many of native fish species have great potential as aquarium fish due to its peculiar body sharp, colorations, swimming behavior, catching easily (and are free), and readily adapting to aquarium life and accepting standard fish food. However, at present, these fish species in the DNBR have not been used to their true value but are considered as trash fish with low economic value. Therefore, an up-to-date study to assess the diversity of native fish species, especially ornamental fish species at the DNBR is necessary to introduce proposed measures to conserve endangered species, protect and develop economic fish species and efficiently use of native ornamental fish species. The outcomes of the study will help local people to develop economy, to improve their livelihoods as well as to raise awareness in the sustainable use of aquatic resources and conservation of these species.

2. Materials and Methods

The study on the diversity of native ornamental fishes in the DNBR was implemented from May 2019 to December 2020.

The samples were collected from different types of water bodies of the typical aquatic ecosystems of the DNBR included Suoi Rang, Suoi Sa Mach, Suoi Da Dung, Suoi Cop, Ba Hau, Tri An Reservoir and Ramsar Bau Sau (Nam Cat Tien National Park). Dip nets, seine nets and fishing trap were used to catch fish because they are the most efficient and productive collection methods with the least negative impacts on the collected samples.

Fish samples were weighed and measured (according to the instructions of Pravdin, 1973). Fish samples were then photographed in the field, labeled with a local name, time, location, inserted labels into the mouth or gill, and stored in 10% formalin solution. Alive fish samples were kept in continuously aerated plastic containers and in the dark and at low temperatures during collecting and transportation. Fish specimens were transferred to the laboratory of Faculty of Fisheries, Nong Lam University (FoF, NLU) for identification. A total of 374 alive individuals of 21 collected species continue to be kept at Freshwater hatcheries of FoF, NLU to monitor the survival, growth, adaptation and reproduction in captivity conditions. The fish were typically separated by species and domesticated in separate tanks (600-L fiberglass tank). *Ceratophyllum* and black pebble stone were equipped into the domesticated tank to simulate the habitat of the fish in the wild. In addition, most of the native species are somewhat timid and have a hiding habit, so PVC pipes were also fitted. The tanks were kept in a quiet place with medium light and lightly aerated. Fish were fed satisfactorily once a day. Fish species liked *Rasbora* sp., *Esomus metallicus, Chela laubuca, Danio pulcher, Betta prima, Nemacheilus platiceps*,... were fed *Moina* and red worms (*Tubifex* sp.), while *Channa* cf. *gachua*, *Pseudomystus siamensis* and *Ompok siluroides*... were fed white spot fish (*Aplocheilus panchax*), small tilapia and freshwater Atyidae shrimp (*Caridina* sp.).

The morphometric and meristic characteristics of fish samples such as total length, standard length, and numbers of dorsal fin, pelvic fin, pectoral fin, lateral line scale, etc. were iden-

tified based on the taxonomic keys published by Vidthayanon (2008), Vasil'eva et al. (2013) and Nelson et al. (2016).

The economic importance and the threat and conservation status were determined according to the Vietnam Red Book (MOST, 2007) and IUCN Red list of Threatened Species (IUCN, 2015).

Ornamental fish were selected based on criteria such as having various and beautiful sizes, shapes and colors, and suitable for keeping as pets in an aquarium, a tank, pond or a container for decorative or display purposes.

3. Results and Discussion

Analytical results recorded a total of 116 fish species belonging to 11 orders and 28 families (Table 1). Of these, the most dominant order was Cypriniformes (50 species, accounting for 43.9%), followed by Perciformes (24 species, 20%) and Siluriformes (21 species, 18.4%) (Figure 1). Out of 116 recorded species, four species of fish

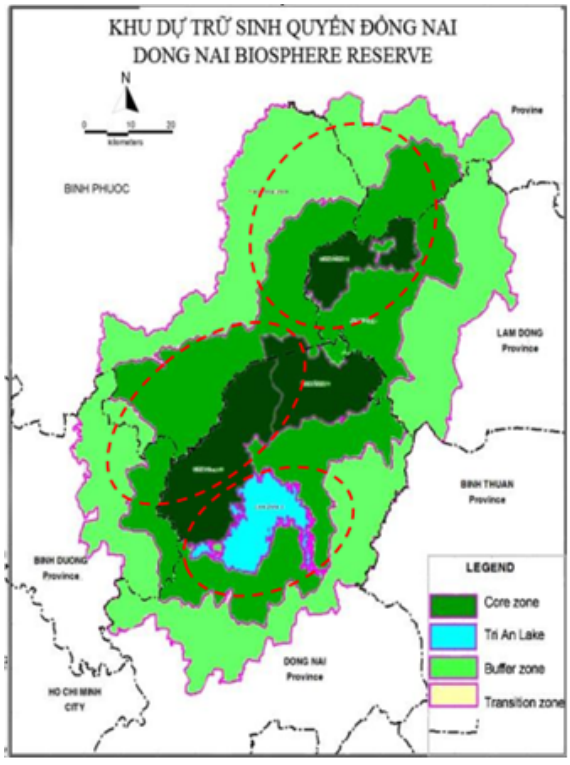

Figure 1. Studied sites at the Dong Nai biosphere reserve.

have been listed under the list of rare and endangered fish species (three species on the Vietnam Red List; one species on the IUCN's Red List), 77 species were considered as food fish. The study revealed the abundant occurrence of 53 native fish species belonging to 7 orders and 15 families in the DNBR (Table 1) that have ornamental value, such as *Nemacheilus platiceps, Lepidocephalichthys hasselti, Rasbora borapetensis, Rasbora trilineata, Gyrinocheilus pennocki, Gyrinocheilus aymonieri, Channa* cf. *gachua, Brachygobius* cf. *nunus, Danio pulcher, Chela laubuca*... These species of fish are generally small in size, but this is what makes them beautiful. The small size also makes them suitable for keeping in aquariums. Among these native ornamental fish species, there are 31 species of food fish at the juvenile stage having special colors and morphology that are valuable as ornamental fish such as *Cyclocheilichthys* cf. *lagleri, Osteochilus lini, Cyclocheilichthys repasson, Mystus nemurus, Mystus singaringan, Xenentodon cancila*... Some of these native fish have been acclimated to artificial environmental conditions of aquariums such as *Chitala ornata, Pseudomystus siamensis, Mastacembelus armatus, Mastacembelus favus, Carinotetraodon lorteri*... Native fish are an excellent alternative to exotic fish species because of their availability, low cost and no devastating impact on the natural aquatic environment. In addition, keeping native fish in an aquarium allows aquarists to recreate a microcosm of their habitat in the wild and observe them at home or workplace. Through observing fish behavior and their interaction with different environmental conditions will help promote the awareness and understanding of aquarists about the beauty, function, and ecological services of the fish as well as how our activities affect the ecology of our environment. From there, it helps to raise awareness about protecting the natural environment, especially the aquatic ecosystem in their living areas (Figure 2).

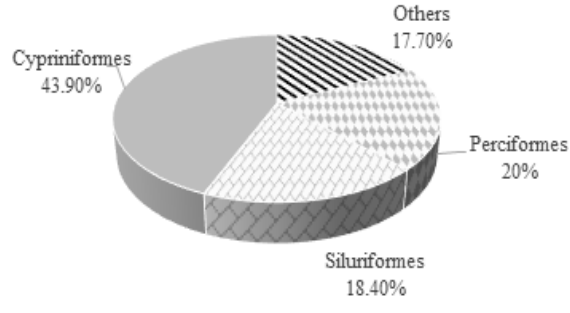

Figure 2. Fish species composition recorded at Dong Nai biosphere reserve.

Table 1. The fish species composition in DNBR, Dong Nai (2020)

	Scientific name	REF			FF	OF
		1	2	3		
	Osteoglossiformes					
	Notopteridae					
1	*Chitala ornata* Gray, 1831	EN	VU	LC	X	X
2	*Notopterus notopterus* Pallas, 1780				X	X
	Clupeiformes					
	Clupeidae					
3	*Corica laciniata* Fowler, 1935				X	
4	*Clupeichthys aesarnensis* Wongratana, 1983				X	
5	*Clupeoides borneensis* Bleeker, 1852				X	
	Cypriniformes					
	Balitoridae					
6	*Nemacheilus platiceps* Kottelat, 1990					X
7	*Acantopsis dialuzoha van* Hasselt, 1823					
8	*Lepidocephalichthys hasselti* Valenciennes, 1846					X
	Cyprinidae					
9	*Barbonymus gonionotus* Bleeker, 1849				X	
10	*Barbonymus schwanenfeldi* Bleeker, 1854				X	X
11	*Barbonymus altus* Gunther, 1868				X	
12	*Cosmochilus harmandi* Sauvage, 1878	VU	VU	LC	X	
13	*Chela laubuca* Hamilton, 1822					X
14	*Cirrhinus microlepis* Sauvage, 1878				X	
15	*Crossocheilus reticulatus* Fowler, 1934				X	X
16	*Danio pulcher* Blyth, 1860					X
17	*Ctenopharyngodon idella* Valenciennes, 1844				X	
18	*Cyclocheilichthys armatus* Valenciennes, 1842				X	
19	*Cyclocheilichthys enoplos* Bleeker, 1849				X	
20	*Cyclocheilichthys repasson* Bleeker, 1853				X	X
21	*Cyclocheilichthys apogon* Valenciennes, 1842				X	X
22	*Cyclocheilichthys* cf. *lagleri* Sontirat, 1989				X	X
23	*Cyprinus carpio* Linnaeus, 1758				X	
24	*Leptobarbus hoevenii* Smith, 1945				X	X
25	*Poropuntius deauratus* Valenciennes, 1842					
26	*Puntius* cf. *brevis* Bleeker, 1806					X
27	*Puntius rhombeus* Kottelat, 2000					
28	*Hampala macrolepidota van* Hasselt, 1823	VU		LC	X	X
29	*Henicorhynchus caudimaculatus* Fowler, 1934				X	
30	*Henicorhynchus lobatus* Smith, 1945				X	
31	*Henicorhynchus siamensis* Sauvage, 1881				X	
32	*Labiobarbus lineatus* Smith, 1945				X	
33	*Labiobarbus siamensis* Sauvage, 1881				X	
34	*Thynnichthys thynnoides* Bleeker, 1852				X	
35	*Hypophthalmichthys molitrix* Valenciennes, 1844				X	
36	*Hypophthalmichthys nobilis* Richardson, 1845				X	
37	*Labeo chrysophekadion* Bleeker, 1849	VU		LC	X	X
38	*Labeo rohita* Hamilton, 1822				X	X
39	*Osteochilus hasselti* Valenciennes, 1842				X	X
40	*Osteochilus lini* Fowler, 1935				X	X
41	*Osteochilus waandersi* Bleeker, 1852				X	X
42	*Osteochilus microcephalus* Valenciennes, 1842			LC	X	X

Table 1. The fish species composition in DNBR, Dong Nai (2020) (continue)

	Scientific name	REF			FF	OF
		1	2	3		
43	*Paralaubuca barroni* Fowler, 1934					
44	*Parachela maculicauda* Smith, 1934					
45	*Puntioplites falcifer* Smith, 1929				X	
46	*Puntioplites proctozysron* Bleeker, 1865				X	
47	*Puntius orphoides* Valenciennes, 1842				X	X
48	*Rasbora trilineata* Steindachner, 1870					X
49	*Esomus metallicus* Ahl, 1923					X
50	*Rasbora tornieri* Ahl, 1922					X
51	*Rasbora paviana* syn. R. *paviei* Tirant, 1885					X
52	*Rasbora borapetensis* Smith, 1934					X
53	*Scaphognathops stejnegeri* Smith, 1931					
54	*Mystacoleucus marginatus* Valenciennes, 1842					
55	*Systomus aurotaeniatus* Tirant, 1885					
	Gyrinocheilidae					
56	*Gyrinocheilus aymonieri* Tirant, 1883	VU		LC	X	
57	*Gyrinocheilus pennocki* Fowler, 1937					
	Siluriformes					
	Bagridae					
58	*Hemibagrus nemurus* Valenciennes, 1839				X	
59	*Hemibagrus wyckioides* Fang & Chaux, 1949		NT	LC	X	
60	*Hemibagrus filamentus* Fang & Chaux, 1949				X	
61	*Mystus albolineatus* Roberts, 1994				X	X
62	*Mystus rhegma* Fowler, 1935				X	X
63	*Mystus mysticetus* Roberts, 1992				X	X
64	*Mystus nemurus* Valenciennes, 1840	VU	VU	DD	X	X
65	*Mystus singaringan* Bleeker, 1846				X	X
66	*Pseudomystus siamensis* Regan, 1913				X	X
	Bagriichthidae					
67	*Bagrichthys obscurus* Ng, 1999				X	
	Clariidae					
68	*Clarias batrachus* Linnaeus, 1758	CR		LC	X	X
69	*Clarias gariepinus* Burchell, 1822				X	
70	*Clarias macrocephalus* Gunther, 1864				X	
	Loricariidae					
71	*Pterygoplichthys disjunctivus* Weber, 1991					
	Pangasiidae					
72	*Pangasius macronema* Bleeker, 1850				X	
	Akysidae					
73	*Akysis maculipinnis* Fowler, 1934					X
	Siluridae					
74	*Micronema bleekeri* Bocourt, 1866				X	
75	*Micronema apogon* Bleeker, 1851				X	
76	*Ompok siluroides* Lacepede, 1803	CR		NT	X	
77	*Kryptopterus* sp.				X	
78	*Wallago attu* Bloch & Schneider, 1801				X	X
	Beloniformes					
	Belonidae					
79	*Xenentodon cancila* Hamilton, 1822				X	X
80	*Xenentodon canciloides* Bleeker, 1854				X	

Table 1. The fish species composition in DNBR, Dong Nai (2020) (continued)

	Scientific name	REF			FF	OF
		1	2	3		
	Hemiramphidae					
81	*Dermogenys siamensis* Fowler, 1934					X
82	*Hyporhamphus limbatus* Valenciennes, 1847				X	
83	*Zenarchopterus ectuntio* Hamilton, 1822				X	
84	*Dermogenys pusilla* Kuhl & van Hasselt, 1823				X	
	Synbranchiformes					
	Mastacembelidae					
85	*Mastacembelus* cf. *circumcinctus* Hora, 1924				X	X
86	*Mastacembelus armatus* Lacepede, 1800				X	X
87	*Macrognathus siamensis* Gunther, 1861				X	X
88	*Mastacembelus favus* Hora, 1923				X	X
	Synbranchidae					
89	*Monopterus albus* Zuiew, 1793				X	
	Perciformes					
	Anabantidae					
90	*Anabas testudineus* Bloch, 1792				X	
	Ambassidae					
91	*Parambassis siamensis* Fowler, 1937					X
92	*Parambassis apogonoides* Bleeker, 1851					
93	*Parambassis wolffi* Bleeker, 1850					
	Channidae					
94	*Channa lucius* Cuvier, 1831					
95	*Channa striata* Bloch, 1793				X	
96	*Channa* cf. *gachua* Hamilton, 1822					X
	Cichlidae					
97	*Cichla ocellaris* Bloch & Schneider, 1801				X	
98	*Oreochromis mossambicus* Peters, 1852				X	
99	Oreochromis niloticus Linnaeus, 1758				X	
100	*Oreochromis red hybrid*				X	
	Eleotridae					
101	*Oxyeleotris marmorata* Bleeker, 1852				X	X
	Gobiidae					
102	*Brachygobius sabanus* Inger, 1958					
103	*Glossogobius aureus* Akihito & Meguro, 1975					
104	*Glossogobius giuris* Hamilton, 1822				X	X
105	*Papuligobius ocellatus* Fowler, 1937				X	
106	*Gobiopterus* cf. *chuno* Hamilton, 1822					
107	*Brachygobius* cf. *nunus* Hamilton, 1822					X
	Helostomatidae					
108	*Helostoma temminckii* Cuvier, 1829				X	
	Pristolepididae					
109	*Pristolepis fasciata* Bleeker, 1851				X	
	Belontiidae					
110	*Trichopodus microlepis* Gunther, 1861					
111	*Trichopsis vittata* Cuvier, 1831					X
112	*Trichopodus trichopterus* Pallas, 1770					X
113	*Betta prima* Kottelat, 1994					X

Table 1. The fish species composition in DNBR, Dong Nai (2020) (continued)

	Scientific name	REF 1	REF 2	REF 3	FF	OF
	Tetrodontiformes					
	Tetraodontidae					
114	*Monotrete leiurus* Bleeker,1850 syn. *Tetraodon leiurus* Bleeker, 1951					
115	*Carinotetraodon lorteri* Tirant, 1885					X
	Atheriniformes					
	Phallostethidae					
116	*Phallostethus* cf. *smithi* Myers, 1928					

REF: Rare and Endangered Fish.
1: MARD (QĐ-82/2008); 2: Vietnam Red list-2007; 3: IUCN-2015.
FF: Food Fish.
OF: Ornamental Fish.

The results of domestication experiment showed that 21 selected native ornamental fish species, belong to 11 families, adapted, and grew well under captive conditions at the Freshwater hatcheries of FoF, NLU (Table 2). The study also recorded complete adaptations of eight fish species, including *Rasbora borapetensis smith, Rasbora trilineata steindachner, Esomus metallicus, Rasbora paviana* syn. *R. paviei, Betta prima, Mastacembelus armatus, Pseudomystus siamensis* and *Channa* cf. *gachua* with evidence that they grew well and reproduced naturally in captivity. Currently, the number of individuals of these species is greatly increased compared to the number of domestications at the beginning. Especially, the 2nd generation of these species have also reached sexual maturity and have spawned the 3rd generation. The importance is that the 3rd generation fish onwards fully met the criteria to be marketed in the aquarium market. The remaining fish species are well adapted and developed well but have not spawned in captivity, which may be due to the small number of individuals, sexual immaturity, inadequate male-female ratio as well as the unsuitable conditions necessary for reproduction. Therefore, it is necessary to have further studies on the reproductive characteristics of these species, then collecting more individuals from the wild for reproduction testing.

According to Teletchea & Fontaine (2014) there is 5 levels of domestication of wild species, with 1 being the least to 5 being the most domesticated. The classification of the domestication levels includes: level 1 (first trials of acclimatization to the culture environment); level 2 (part of the live cycle closed in captivity, also known as capture-based aquaculture); level 3 (entire life cycle closed in captivity with wild inputs); level 4 (entire life cycle closed in captivity without wild inputs); and level 5 (selective breeding programmes are used focusing on specific goals). Based on this classification, it is clear that eight species recorded in this study, *Rasbora borapetensis smith, Rasbora trilineata steindachner, Esomus metallicus, Rasbora paviana* syn. *R. paviei, Betta prima, Mastacembelus armatus, Pseudomystus siamensis* and *Channa* cf. *gachua*, are at level 4 of domestication meaning truly domesticated. While the 13 species actually belong to the first three levels of domestication, implying that the current trade of these species is based on entirely or partly on wild catch. Therefore, these species need to be further domesticated before being marketed.

The survey results showed that freshwater ornamental fish accounted for about 99% of Vietnam's total ornamental fish exports in 2019, of which marine species contributed less than 1%. Out of the total of approximately 60 freshwater ornamental fish species being exported from Vietnam, there are approximately 50 artificial reproduction species and about 10 wild fish species (PSN, 2020). As of October 2019, Ho Chi Minh City's ornamental fish production reached 176 million fish, of which the export volume was 17.9 million fish. Export value reached 19,66 million USD. Ho Chi Minh City's ornamental fish has been exported to 50 countries of which Europe accounted for 54.1%, Asia accounted for 28.6% and America accounted for 14.5% (Chi, 2020). It indicated that ornamental fishes have a significantly important market of high demand and prices both in domestic market and in international markets. The results showed that many of the founded species in this study are

Table 2. The fish species are being domesticated at the Freshwater hatcheries of Faculty of Fisheries, Nong Lam University

	Scientific name	Adapted to captivity	Natural reproduction in captivity
	Cyprinidae		
1	*Chela laubuca* Hamilton, 1822	X	
2	*Danio pulcher* Blyth, 1860	X	
3	*Puntius rhombeus* Kottelat, 2000	X	
4	*Paralaubuca barroni* Fowler, 1934	X	
5	*Puntius orphoides* Valenciennes, 1842	X	
6	*Rasbora trilineata* Steindachner, 1870	X	X
7	*Esomus metallicus* Ahl, 1923	X	X
8	*Rasbora paviana* syn. *R. paviei* Tirant, 1885	X	X
9	*Rasbora borapetensis* Smith, 1934	X	X
	Bagridae		
10	*Pseudomystus siamensis* Regan, 1913	X	
	Akysidae		
11	*Akysis maculipinnis* Fowler, 1934	X	
	Siluridae		
12	*Ompok siluroides* Lacepede, 1803	X	
	Belonidae		
13	*Xenentodon cancila* Hamilton, 822	X	
	Channidae		
14	*Channa* cf. *gachua* Hamilton, 1822	X	X
	Belontiidae		
15	*Betta prima* Kottelat, 1994		
	Mastacembelidae		
16	*Mastacembelus* cf. *circumcinctus* Hora, 1924	X	
17	*Mastacembelus armatus* Lacepede, 1800	X	X
18	*Macrognathus siamensis* Gunther, 1861	X	
19	*Mastacembelus favus* Hora, 1923	X	X
	Tetraodontidae		
20	*Carinotetraodon lorteri* Tirant, 1885	X	
	Gobiidae		
21	*Brachygobius* cf. *nunus* Hamilton, 1822	X	
	Balitoridae		
22	*Nemacheilus platiceps* Kottelat, 1990	X	

on the list of the main export freshwater ornamental species of Vietnam, or have similar shapes and colors with these species. Information from aquarium trade websites shows that fish species found in this study such as *Brachygobius doriae, Rasbora borapetensis, Rasbora trilineata, Leiocassis siamenses, Pseudomystus siamensis, Carinotetraodon lorteri, Mastacembelus armatus, Mastacembelus favus...* are being exported to many countries around the world. However, the main supply of these species is still dependent on the wild-caught. As a result, native ornamental fish species are being harvested from the wild in greater numbers and at higher rates, although many of these species have been domesticated and successfully artificial reproduction. Many studies revealed that fishing natives for the ornamental fish market is threatening the sustainability of aquatic ecosystems and fisheries (Chao et al., 2001; Vagelli & Erdmann, 2002; Lunn & Moreau, 2004). Fortunately, most of the sampling areas in this study are located in the protected area of the DNBR, so the native ornamental fish species are still abundant and has not been harvested for economic purposes. There are many beautiful endemic species and are not yet available on the ornamental market such as *Betta prima, Xenentodon cancila, Channa* cf.

gachua, Akysis maculipinnis, Gyrinocheilus pennocki. Conversely, in other water bodies of the DNBR such as Tri An Lake and Ba Hao Lake, these native species are collected as by-catch of fisheries and sold for much less than their true value, called trash fish. There are three terms for trash fish in Vietnamese: trash fish, trawler fish and pig fish. Trash fish and trawler fish can be used as human food but are rarely used, while pig fish is the least quality and often used as aquaculture feed, livestock feed. Survey results showed that at aquarium shops in Ho Chi Minh city, *Rasbora borapetensis* and *Rasbora trilineata* were sold with average price of 0,3 USD/fish (average weight 3 g/fish), equivalent to 90 USD/kg). However, at local market, it was sold as human food with the price of 3 USD/kg and even lower, less than 1 USD/kg, when used as feed for aquaculture and livestock husbandry.

The survey results also showed that the majority of people living in the DNBR have a relatively low standard of living. Fisheries are an important source of livelihood for many local people. However, the income from fisheries is often low and unstable due to low catches and low value of harvested fish. Therefore, collecting wild fish for ornamental purposes besides fishing food fish can be an alternative for local people. Many researchers agreed that if managed sustainably, the collection of wild fish for the ornamental fish market could provide stable income jobs in predominantly rural, low-income communities (Kiron & Dhanasiri, 2011; Rhyne et al. 2012; Murray & Watson, 2014). To obtain this purpose, the local government must have complete data/information about native ornamental fish species, their status and distribution areas. On that basis, local authorities could provide local people with necessary information about these species including images, market demand, commercial size, and value... Moreover, the local authority needs to issue regulations on fishing such as fishing grounds, seasons, sizes, and catches. In addition, training programs/instructions on the methods of fishing, transportation, and acclimatization that ensuring the captured fish for ornamental purpose are alive and in good health, are essential for local communities. However, in the near future, there is a need to have a plan to domesticate, artificial reproduce and farm of these native ornamental fish species. This means that these fish should reach level 4 of domestication. If these goals are achieved, it will not only help to increase income and livelihood of local people, but also helps prevent over-fishing, helps to conserve rare and critically endangered species as well as help to protect and sustainably use these aquatic resources.

4. Conclusions

Out of 116 fish species recorded in the Dong Nai Biosphere Reserve, there are were four species of rare and endangered fish; 21 species of ornamental fish. Eight species of ornamental species reach level 4 of domestication meaning truly domesticated, while the remaining 13 species belong to the first three levels of domestication, implying that these species need to be further domesticated before being marketed.

If managed sustainably, the collection of wild fish for the ornamental fish market could provide a stable income and livelihood for communities in the Dong Nai biosphere reserve.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Evaluation of drought on agricultural land-use change: A case study of coastal districts, Ben Tre province

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ABSTRACT

Ben Tre is a coastal province in the Mekong Delta heavily affected by negative impacts of climate change and sea level rise, such as freshwater shortage and increased salinity intrusion during the dry season. This research aimed to develop a remote sensing approach, using time series data to assess drought development for the coastal districts (Ba Tri, Binh Dai, and Thanh Phu) in Ben Tre province. The Temperature Vegetation Dryness Index (TVDI) was analyzed based on the time-series Landsat 8 OLI data, which were obtained continuously from 2009 - 2019 to evaluate drought changes over time. The drought maps of 2009 and 2019 were established and the results showed that there were four levels of drought, including non-drought, slight drought, moderate drought and severe drought. Areas with non-drought and slight drought were reported at 5.65% and 35.34% (about 6,098 ha and 38,146 ha), respectively; while about 53.14% and 5.87% of the study areas were classified as moderate and severe drought (about 57,354 ha and 6,332 ha), respectively. The assessment of fluctuations in the period 2009-2019 showed that the areas of non-drought and slight drought tended to decrease while the areas of moderate and severe drought increased. The drought was positively related to agricultural land-use change as shown by the following formula $\log_e(P_i/(1 - P_i)) = 7.985 * TVDI - 6.746$. Drought tended to decrease in the areas where the bare land was changed to lands for perennial crops, rice crops and aquaculture, while drought tended to increase in land-use types of rice and annual crops.

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1. Introduction

Drought is defined as a prolonged period of lack of rainfall, resulting in severe aridity during the dry season (Wilhite & Glantz, 1985). According to the World Meteorological Organization (WMO), droughts are classified into four types: meteorological drought, agricultural drought, hydrological drought and socio-economic drought. The study only focused on agricultural drought. Agricultural drought links various characteristics of meteorological (or hydrological) drought to agricultural impacts, focusing on precipitation shortages, differences between actual and potential evapotranspiration, soil water deficits, reduced groundwater or reservoir levels, and so forth. According to the report of the Institute of Risk Analysis Mapleocroft (England, 10/2010), Vietnam ranks 13 out of 16 countries strongly affected by drought. Drought can have a significant impact on the ecology and agriculture of the affected area, promoting land-use conversion and changing land cover (Raja et al., 2013).

There are many methods to assess drought, but

two common methods are used including: (1) The method of calculating drought index from hydrometeorological station data based on two main meteorological indicators such as the amount of water evaporation and rainfall; (2) Remote sensing method based on the drought index according to the temperature - vegetation relationship. The use of remote sensing for drought monitoring has the advantage of being able to receive regular and continuous information on land surface characteristics in space and time (Belal et al., 2014).

The indicators developed from remote sensing data such as Normalized Difference Vegetation Index (NDVI), Land Surface Temperature (LST) are used to monitor crop growth in agriculture (Han et al., 2010). Wang et al. (2003) developed the Temperature – Vegetation Dryness Index (TVDI) based on information from the LST scatter plot compared to the number of NDVI in a triangle space is created by "wet edge" and "dry edge". The Standardized Precipitation Index (SPI) is the most widely used indicator of drought using meteorological data. SPI can be computed at different time scales and thus can quantify water deficit at different time intervals. SPI is designed to demonstrate that it is possible to experience wet conditions simultaneously over one or more dry sand conditions on a different time scale (Jain et al., 2010). Standardized Water Supply Index (SWSI) is a hydrological drought index developed to replace the Palmer Drought Severity Index (PDSI) in areas where local precipitation is not the only (or primary) source of runoff. SWSI is calculated based on monthly non-exceeded probabilities determined using historical data information available on reservoir storage, runoff flow, precipitation, and ice (Hayes et al., 2012).

In Vietnam, there are many studies on the application of GIS and remote sensing in drought risk assessment including the study of Bui et al. (2019) in Tuong Duong district, Nghe An province, using the SAVI and TVDI indexes to assess drought based on multi-spectrum satellite images. Comparing with local survey and statistics, the results calculated according to TVDI index reflect the term more accurately, in detail and more closely than the SAVI index. Therefore, TVDI is more suitable and effective in assessing drought in Tuong Duong (Bui et al., 2019). The study of the authors Trinh & Dao (2015) used the TVDI index to assess the risk of drought for Bac Binh district, Binh Thuan province. Research results show that most of Bac Binh district is forecasted to have moderate to severe and extreme drought, in which areas at risk of severe and extreme drought increase very rapidly in the years 2010, 2014 compared to previous years. Trinh (2014) studied soil moisture and the degree of dryness of the land cover based on the Temperature Vegetation Dryness Index (TVDI) using thermal image data LANDSAT TM, ETM +, LANDSAT 8 OLI.

Ben Tre is one of the provinces heavily affected by drought and saltwater intrusion, especially in the coastal districts of Ba Tri, Binh Dai and Thanh Phu, where the outlet of the main rivers has very different types of land use sensitive to drought and saltwater intrusion such as rice land and aquacultural land (Figure 1). This paper introduces the application of remote sensing in drought zoning and evaluates the effects of drought on agricultural land use change in coastal districts of Ben Tre province.

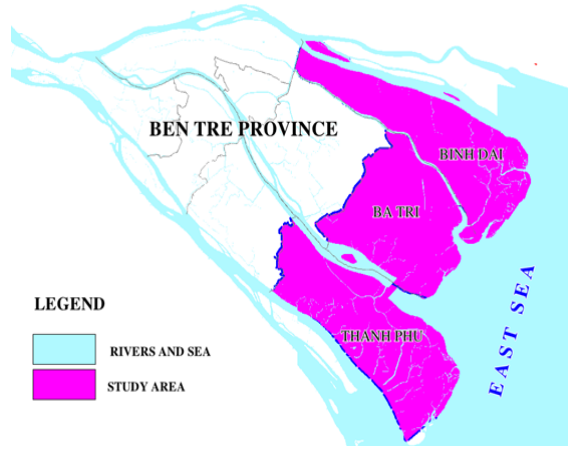

Figure 1. Location of the study area.

2. Materials and Methods

2.1. Materials

Satellite images (Landsat 7 and Landsat 8) are used for land use mapping, surface temperature maps, and vegetation growth index maps. Thematic maps are used to assist in sampling and classifying images for land use types mapping. Socio-economic and land statistics in 2019 data are used in the assessment of socioeconomic and land use status. Field survey data used to assess

drought.

2.2. Research methods

2.2.1. Images classification method

With the input data source is Landsat 7,8 satellite image, the study uses the Maximum Likelihood Classification method (MLC). MLC is a supervised image classification method based on Bayes theorem that is considered to have high accuracy and is widely used. The MLC algorithm will consider each class in each spectral channel to have a normal distribution. The pixels will be assigned to the category that has the highest probability. The calculation is not only based on the value of the spectral distance, but also on the trend of brightness variation in each type.

2.2.2. The method of calculating the drought index from remote sensing images

The remote sensing method for drought zonning based on the drought index according to the vegetation and temperature relationship (Temperature Vegetation Dryness Index – TVDI). The TVDI is calculated using the following equation:

$$TVDI = ((T_s - T_{smin})/((T_{smax} - T_{smin})))$$

$$= ((T_s - T_{smin})/((a + b * NDVI - T_{smin})))$$

where T_{smin} is the minimum surface temperature in the triangle to determine the wet edge; Ts is the observed temperature at the image pixel to be calculated; Tsmax is the maximum observed surface temperature for each range of NDVI values. The parameters a and b of the "dry edge" line for a Landsat image are determined by the least squares regression function of the maximum values Ts for the NDVI ranges. The parameters a, b are coefficients of the following equation:

$$T_{smax} = a + b * NDVI$$

Where: NDVI = (NIR - RED)/(NIR_RED)

To determine the coefficients a and b in the above linear function, it is necessary to select the sample points that are the points with the center value of the divided values of the independent variable NDVI and the temperature value corresponding to the dependent variable temperature surface LST. TVDI index received the value from 0 to 1. Classification of surface drought level for TVDI is presented in Table 1.

Table 1. TVDI drought index classification[1]

No	TVDI ranges	Drought levels
1	0.0 - 0.4	No drought
2	0.4 - 0.6	Slight drought
3	0.6 - 0.8	Moderate drought
4	0.8 - 1.0	Severe drought

[1]TVDI: Temperature Vegetation Dryness Index

2.2.3. Statistical method

Includes descriptive statistics and binary logistic regression to determine the correlation between LST and NDVI, degree of drought to land use change. Regression analysis is to find the dependence of one variable, called the dependent variable on one or more other variables, called the independent variable, to estimate or predict the expected value of the dependent variable when knowing the value of the independent variable. In this study, the dependent variable is determined as land use change (with the value 0 being unchanged and 1 being changed) and the independent variable is the degree of aridity calculated by the TVDI index value (with value from 0 to 1).

2.2.4. Research process

To assess the impact of drought on agricultural land use change, the study used Landsat images, thematic maps combined with field surveys (Figure 2).

Bands 4,5 of Landsat images are used to generate NDVI maps, band 10 is used to generate land surface temperature (LST) maps. The drought map is created from the correlation relationship between LST and NDVI. Bands 4,5,7 are used to interpret images and create land use maps in 2009 and 2019. From land use maps and drought maps to assess the effects of drought on agricultural land use changes.

3. Results and Discussion

3.1. Characteristics of natural conditions

Ben Tre province has a natural area of 2,360 km^2. There are coordinates from 9°48' – 10°20' North latitude to 105°57' – 106°48' East longitude. Ben Tre's topography is flat, with mangroves along the coast and rivers. Ben Tre is located in the sub-equatorial monsoon tropical

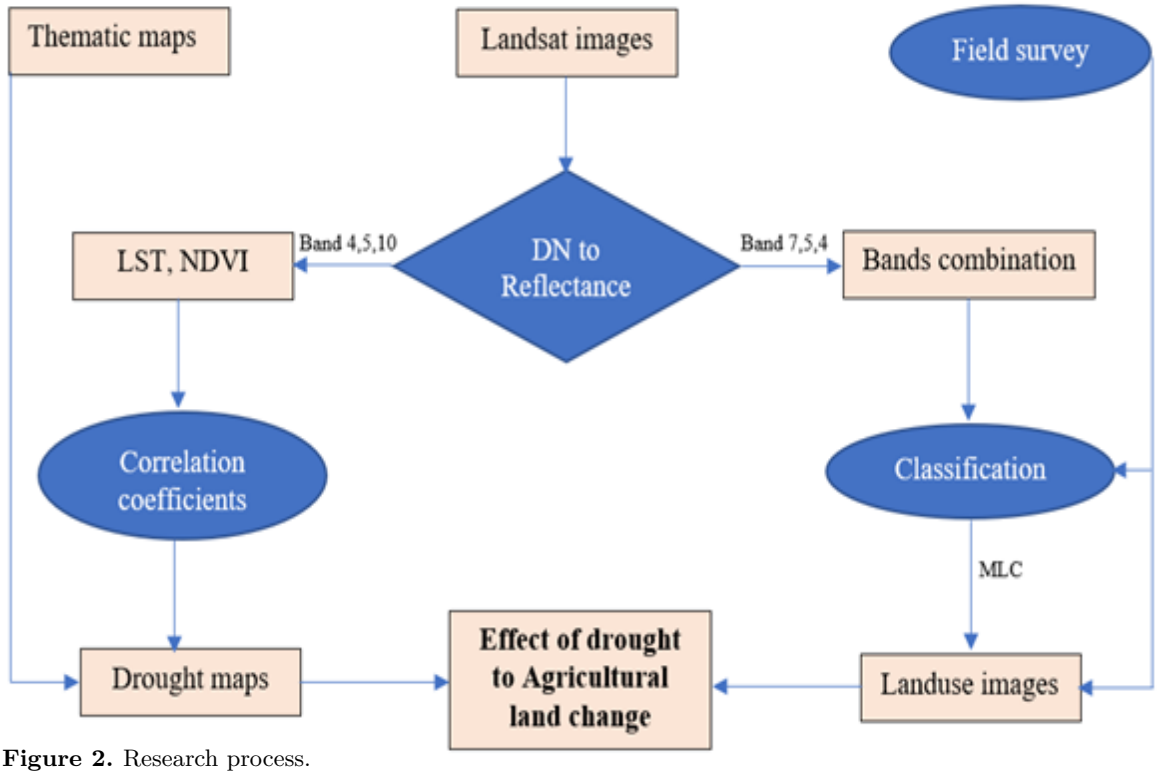

Figure 2. Research process.

climate with annual average rainfall from 2,000 to 2,300 mm, average annual temperature from 26°C to 27°C. According to Ben Tre province's land degradation synthesis report in 2019, there were 5 main soil groups in Ben Tre province, including Artificial (man-made) soil 94,359 ha, saline soil 58,173 ha, alluvial soil 25,225 ha, and alkaline soil 9,915 ha and sandy soil 15,233 ha, of which mainly man-made and saline soils. The current state of land use in 2019 showed that the agricultural land group had an area of 181,821 ha, of which was mainly aquaculture land, followed by land for perennial crops, land for annual crops (mainly rice land). Non-agricultural land group 57,180 ha includes main types of land such as water bodies, specialized land, residential land and other types of non-agricultural land. Unused land group 433 ha is unused flat land. Due to the characteristics of natural conditions, the flow from the headwaters of the Mekong River to the delta is rapidly decreasing and is at a very low level compared to the average document for many years from 1980 to now, the prolonged lack of rain combined with the use and exploitation of water resources in the basin (increasing water use on tributaries and storing water in dams) will make droughts, water shortages, and saltwater intrusion more prolonged and severe in the coastal districts of Ben Tre province.

3.2. Land use/land cover change evaluation in the period of 2009 - 2019

3.2.1. Images classification

The maximum likelihood classification (MLC) method was applied with 115 sample points and 25,460 training pixels selected. Five land use types were classified including rice land, perennial land, aquaculture land, residential land and water bodies. The image classification results show that rice land is concentrated in Ba Tri district where there is Kenh Lap freshwater lake with an area of 151 ha; perennial land is concentrated in the northeast of the districts where it is adjacent to coconut and fruit trees of Ben Tre province; aquaculture land distributed along the coast and main rivers; residential land is distributed in urban centers of districts and along traffic roads; water bodies are the main rivers. The area of the land use/land cover types is shown in Table 2 and the distribution of land use/land cover types is shown in Figure 3.

Classification accuracy was assessed by random

Table 2. Land use/Land cover area of 2009, 2019

Land use/Land cover	2009 (ha)	2019 (ha)	Change (ha)
Rice land	12,725	3,747	-8,979
Perennial land	33,754	37,737	3,984
Aquaculture land	45,305	48,379	3,075
Residential land	7,923	9,843	1,921
Water bodies	8,222	8,222	0
Total	107,929	107,929	0

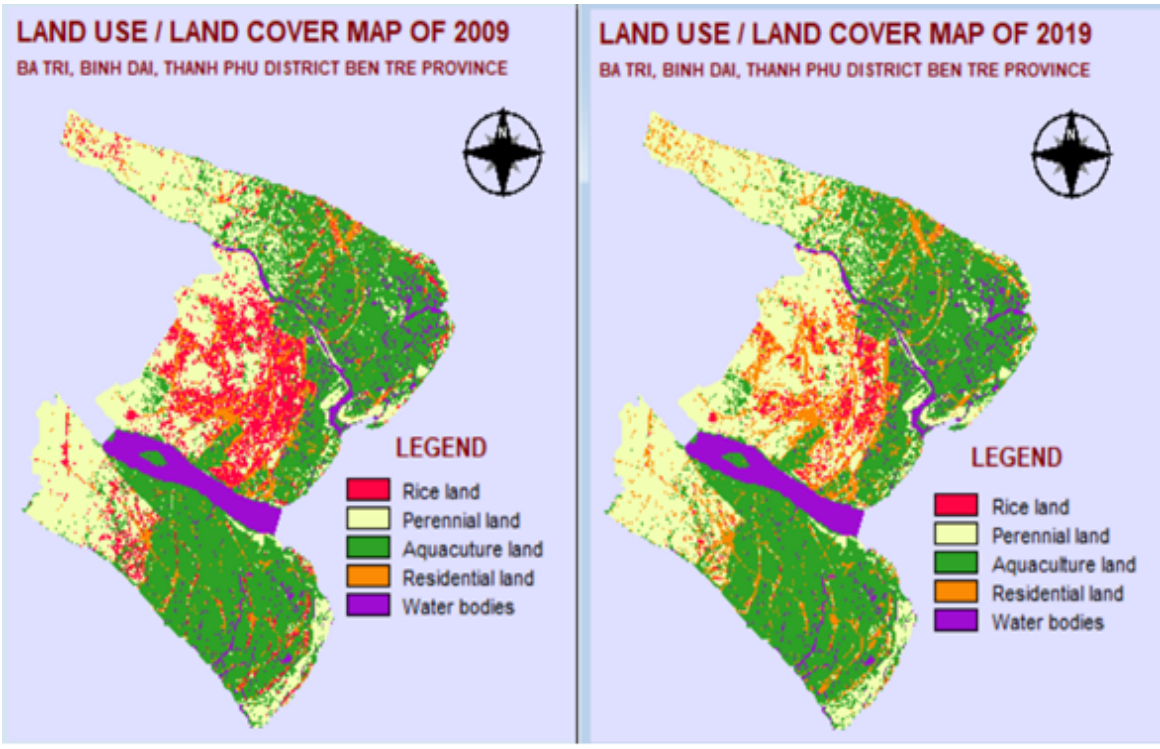

Figure 3. Image classification of 2009 and 2019.

sampling. Pixels belonging to the sample regions (ROIs) of each class are called sample pixels or trained pixels. High-resolution Google earth images combined with land use maps of the study area are used both as a basis for sample zoning and to check classification results. Kappa index and overall accuracy of 2009 images are 0.79 and 83%, respectively; corresponding to the 2019 image is 0.91 and 93%.

3.2.2. Land use/land cover change detection in the period of 2009 - 2019

From the 2009 and 2019 land use / land cover maps, a change assessment was carried out to calculate the changing area and the probability of conversion between types of land use / land cover. The change of area in the period 2009 - 2019 is shown in Figure 4.

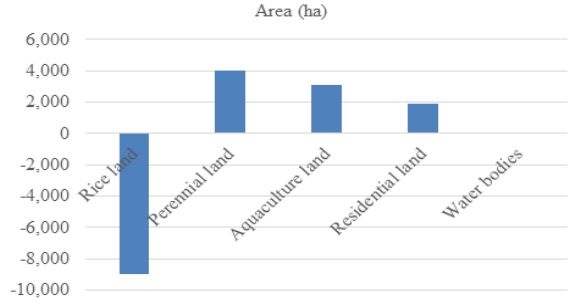

Figure 4. Change area in the period 2009 – 2019.

The results of the assessment of changes in the period 2009 - 2019 showed that the area of rice

land decreased by 8,979 ha, of which the area changed to perennial land 3,984 ha, changed to aquaculture land 3,075 ha, converted to residential land 1,921 ha. The transition probabilities between land use / land cover types are shown in the following map (Figure 5):

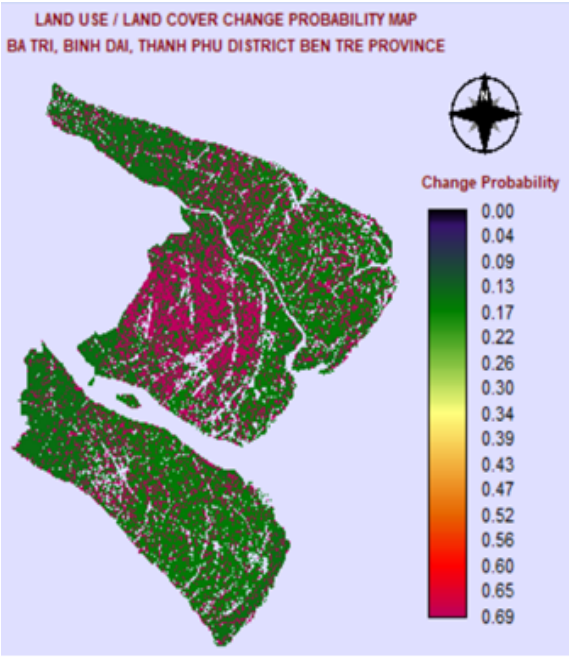

Figure 5. Land use/Land cover change probability.

From the change analysis results, it shows that the changeability of rice land is the highest (69%), aquaculture land and perennial land has little change (from 10 - 20%) while residential land and water bodies hardly change (0%).

3.3. Establishing drought maps of 2009 and 2019

To create a drought map, it is necessary to use band 10 (thermal band) to calculate LST, band 4,5 of Landsat 8 images to calculate the NDVI index. Sample points are selected to calculate the correlation coefficient between LST and NDVI. The coefficients in the correlation equation will be used to create the TVDI index map. From the TVDI index map, a map of drought zoning will be created. The steps to create a drought zoning map are shown in Figure 6.

$$TVDI = ((T_s - T_{smin})/((T_{smax} - T_{smin})))$$
$$= ((T_s - T_{smin})/((a + b * NDVI - T_{smin})))$$

Where: $T_{smax} = a + b * NDVI$

To determine the coefficients a and b in the above linear function, it is necessary to select the sample points that are the points with the center value of the divided values of the independent variable NDVI and the temperature value corresponding to the dependent variable temperature surface LST.

From the above linear equation determine the coefficients a and b, respectively a = -11.975 and b = 27.291 (Figrue 7). T_{smin} and T_{smax} are the highest and lowest temperatures of the surface temperature (LST) map made above, respectively Tsmin = 21°C and Tsmax = 39°C. Substituting the above coefficients into the equation we have:

$$TVDI = (T_s - 21)/(27.291 * NDVI - 32.975)$$

From the above equation, it can be seen that the TVDI drought index is calculated according to the relationship between LST and NDVI index. Using the Band Math function in Envi 5.2 software with two images, LST in 2009, 2019 and NDVI in 2009, 2019 to create a TVDI index map in 2009, 2019. Based on the table of TVDI value classification (Table 3), to establish drought zoning maps for the study area.

The 2019 drought zoning map shows that there are four levels of drought, which are mainly non-drought and slight drought areas in rice and aquaculture areas, distributed in all three districts, in which most of them are concentrated in Thanh Phu district. The moderate drought area is actively irrigated, so the drought level of the soil does not affect the crops because the soil is increased moisture by irrigation. The area of severe drought is scatteredly distributed in the districts with an insignificant area (Figure 8).

Statistical results of fluctuations in the period 2009 - 2019 show that the non drought and slight drought areas tend to decrease while the moderate and severe drought areas increase. The following are some drought images from the field survey results used to verify and correct the drought map (Figure 9 và 10).

3.4. Assessing the impact of drought on agricultural land use change

Conduct a drought value survey at 1,000 sample points to determine correlation with land use change. The value of land use change is recorded in two states of change (value is 1) and unchanged (value is 0). Applying the binary logistics regres-

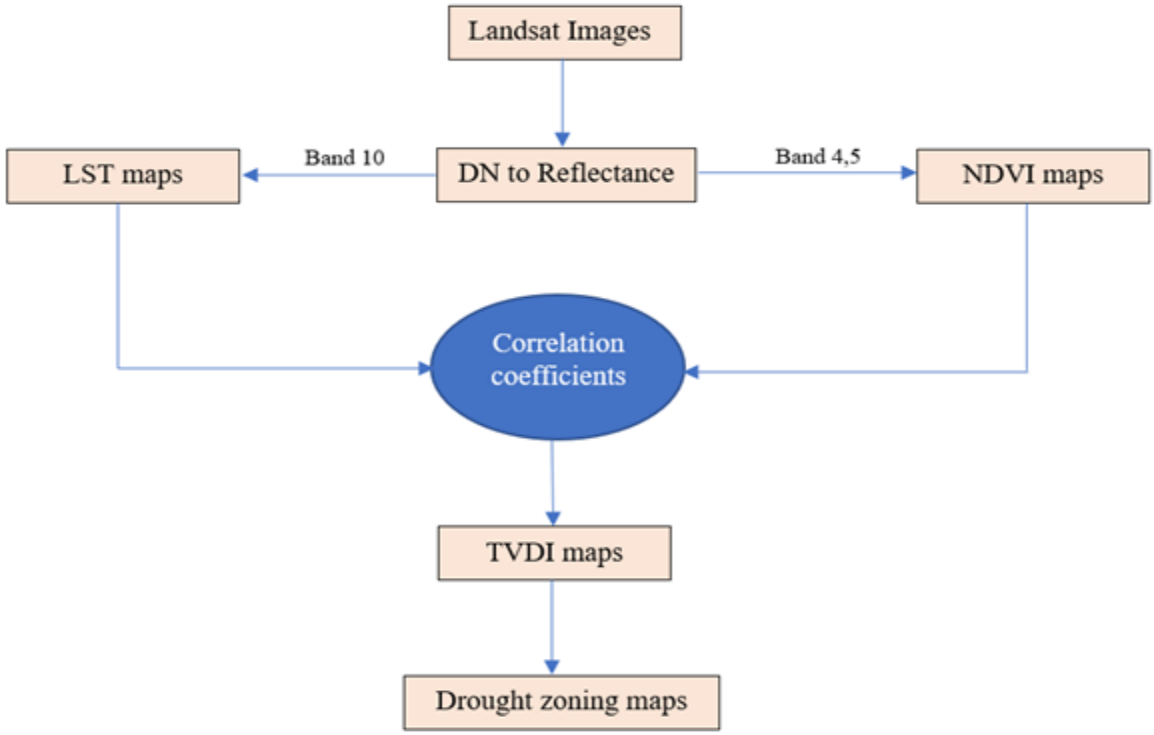

Figure 6. The process of creating drought zoning maps.

Table 3. Statistics of drought areas in 2009, 2019

Drought levels	Area 2009 (ha)	Ratio (%)	Area 2019 (ha)	Ratio (%)	Change 2009 - 2019 (ha)
Non-drought	5,317.83	4.93	6,097.59	5.65	779.76
Slight drought	50,753.43	47.02	38,145.87	35.34	-12,607.56
Moderate drought	47,539.26	44.05	57,354.48	53.14	9,815.22
Severe drought	4,319.37	4.00	6,331.95	5.87	2,012.58
Total	107,929.89	100.00	107,929.89	100.00	0.00

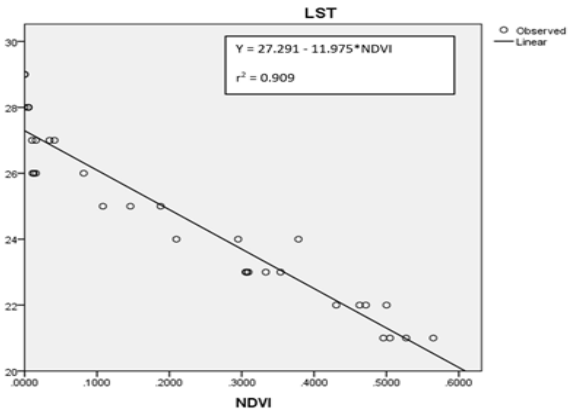

Figure 7. Correlation between LST and NDVI.

sion technique with the following results (Table 4):

Table 4. Omnibus tests of model coefficients

		Chi-square	df	Sig.
Step 1	Step	102.055	1	.000
	Block	102.055	1	.000
	Model	102.055	1	.000

The Omnibus Tests of Model Coefficients table shows that the sig of all 3 Step Block Model indexes is equal to 0.000 < 0.05 (95% confidence level), so the regression model is statistically significant.

Statistical results showed that: In 639 cases where land use did not changed, it is predicted that 537 cases do not change. The correct predic-

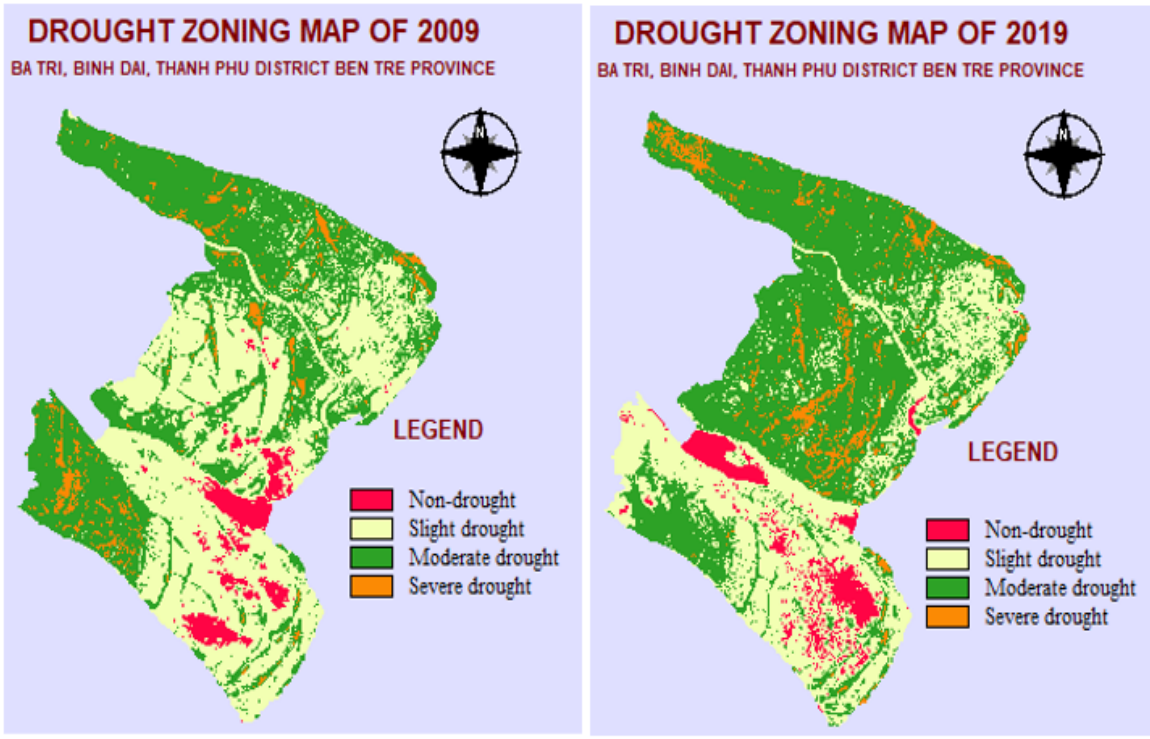

Figure 8. Drought zoning maps of 2009, 2019.

Figure 9. Drought image at Ba Tri.

tion rate was 84.0%. Out of 361 cases of observed land use change, 118 cases of land use change are predicted. The correct prediction rate was 32.7%. Thus, the average rate of correct prediction was (84.0 + 32.7)/2 = 65.5%.

From the results (Table 5), the regression equation has the following form:

$\log_e(P_i/(1 - P_i))=7.985 * TVDI - 6.746$

The results of the regression analysis show that drought is positively related to agricultural land

Figure 10. Drought image at Binh Dai.

Table 5. Variables in the equation

		B	S.E.	Wald	df	Sig.	Exp(B)
Step 1[a]	Drought	7.985	.866	84.997	1	.000	2.817
	Constant	-6.746	.681	98.035	1	.000	.001

a. Variable(s) entered on step 1: Drought.

use change. That is, the more drought areas are, the higher the probability of agricultural land use change and vice versa.

The map of drought change in the period 2009 - 2019 was established with three states: The decreased degree of drought (pixels with TVDI index in 2019 was smaller than TVDI index in 2009); Unchange drought (pixels have TVDI values unchanged between 2009 and 2019) and drought tends to increase (pixels have TVDI values in 2019 was greater than TVDI values in 2009) (Figure 11).

The results of zoning drought fluctuations in Ba Tri, Binh Dai and Thanh Phu districts of Ben Tre province show that the drought area reduced by 5,399 ha, concentrated in Thanh Phu district where formerly bare land and annual crops were transferred to perennial land and rice land. The area does not change 43,982 ha, concentrated in Thanh Phu and Binh Dai districts where the water surface for aquaculture is located. The drought area increased by 58,548 ha distributed in all three districts but mainly concentrated in

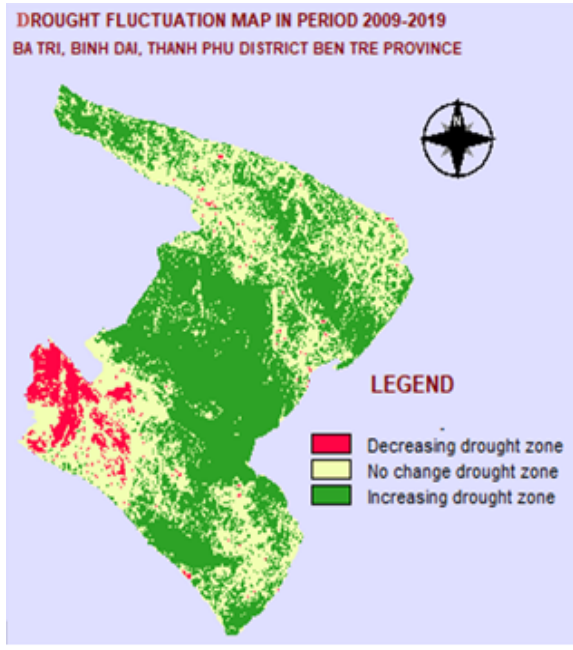

Figure 11. Drought fluctuation in the period 2009 - 2019.

Ba Tri district. Evaluation of the effect of drought on agricultural land use change showed that increased drought leads to changes in annual land and rice land. Drought is reduced in bare land and annual land where they tend to be converted to perennials and aquaculture land. While land use change between types of land for perennial crops and aquaculture land, drought did not change.

4. Conclusions

The coastal districts of Ben Tre province are vulnerable to drought and saltwater intrusion caused by characteristics of natural conditions. The image classification results with five land use types showed that rice land is concentrated in Ba Tri district where there is Kenh Lap freshwater lake; perennial land is concentrated in the northeast of the districts where it is adjacent to coconut and fruit trees of Ben Tre province; aquaculture land distributed along the coast and main rivers; residential land is distributed in urban centers of districts and along traffic roads; water bodies are the main rivers. The drought maps of 2009 and 2019 were established showing that there are four levels of drought, including non-drought, slight drought, moderate drought and severe drought. Areas with non-drought and slight drought were reported at 5.65% and 35.34%, respectively (about 6,098 ha and 38,146 ha); while about 53.14% and 5.87% of the study areas were under moderate and severe drought, respectively (about 57,354 ha and 6,332 ha). The assessment of fluctuations in the period 2009 - 2019 shows that the areas of non-drought and slight drought tend to decrease while the areas of moderate and severe drought increase. There was a positive correlation between drought and agricultural land use change. Drought tended to decrease in the areas where the bare land is changed to lands for perennial crops, rice crops and aquaculture, while drought tended to increase in land use types of rice and annual crops.

Conflict of interest

The authors declare no conflict of interest.

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Hydrolysis conditions of black bean and brown rice and application of the hydrolysate in trial production of plant-based milk

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ABSTRACT

Brown rice and black beans were known as good food sources. The main objective of this study was to evaluate the physicochemical and antioxidative properties of brown rice and black bean extracts treated with different enzymes such as Bialfa-T, Glucozyme 2x, Bialfa-T-Glucozyme 2x, Biase, Biomatasa-L andBiase-Biomatasa-L. These extracts were initially used to produce plant-based milks. The results showed that the combination of Bialfa-T and Glucozyme enzymes expanded the recovery yield and total phenolic content. Combined Bialfa-T and Glucozyme enzymes also resulted in the highest antioxidant capacity in both brown rice and black beans extracts. The product made from the mixture of brown rice extract and black bean extract at a ratio of 1:1 (v/v) had an acceptableorganoleptic quality.

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1. Introduction

Nowadays, people increasingly tend to use plant-based food as an alternative source of animal products (Mäkinen et al., 2016) due to the fear of chronic diseases such as lactose intolerance, diabetes, cardiovascular, etc. Besides that, plant-based food are selected for dietary needs and religious issues. Plant milk is the water extracts of legumes, oilseeds, and cereals (Zandona et al., 2020). According to the information reviewed by Hayat et al. (2014), black beans and common beans are excellent sources of dietary nutrition such as protein, fiber, minerals (iron, zinc, copper, phosphorous, and aluminum), vitamin B complex. Moreover, the beans have a low glycemic index, low lipid content. The major bioactive compounds reported in beans are phenolic compounds (ferulic acid, p-coumaric acid, and gallic acid), flavonoids (kaempferol, quercetin, catechin, and proanthocyanidin), and anthocyanins (3-O-glucosides of malvidin, petunidin). Utilization bean help protect the consumer health by preventing disease such as cardiovascular, obesity, diabetes, etc. Rice (*Oryza sativa*) is a staple food and is widely cultivated all over the world. Brown rice is whole rice, which only the husk is removed. The brown rice was found to have a greater content of nutrients such as protein, lipids, minerals (calcium, sodium, potassium, and vitamins (B complex, E) than the refined rice. Besides, brown rice contains bioactive compounds such as phenolic compounds, anthocyanins, especially γ-oryzanols, GABA. Many

scientific reports show brown have the health benefits such as antioxidant activity, antidiabetic activity, antiobesity and cholesterol-lowering activity, etc (Saleh et al., 2019). Mixing brown rice and black bean in the diet can provide a balance of the biological value of the two materials (Dos Santos et al., 1979).

In Vietnam, processed products from black beans or brown rice are still not diversified. They are mostly used as homemade milk or instant roasted powders. At present, combined milk from these two materials is not available. If the extraction relies only on the ability of water to play as a solvent for the solubility of the solutes in the raw material, the recovery yield of bioactive compounds is not high. In many cases, using enzymes such as amylase for hydrolyzing both the cotyledon and the aleuron layer of the grains could result in higher extraction performance for these essential nutrients in the final product (Xu et al., 2015; Zeng et al., 2018). This study aimed to evaluate the enzyme ability to improve the quality of the black bean and brown rice extract (recovery yield, bioactive compound), as well as produce a non-dairy milk product from the combination of brown rice and black bean.

2. Material and Methods

2.1. Material and chemical

2.1.1. Material

The black bean used in this study was supplied from Phu Minh Tam Company (Vietnam). The beans had black outer skin and green inner cotyledon, with no presence of termites and a moisture content of 17%. Brown rice with a moisture content of 16% and free of termites was supplied from Kim Thien Loc Company (Vietnam).

2.1.2. Chemical

In this study, the used chemicals and reagents included 1,1-Diphenyl-2-picrylhydrazyl (DPPH) with free radical > 97% (HPLC grade) from TCI (Japan); Folin-Ciocalteus reagent from Merck (Germany; gallic acid), sodium carbonate anhydrous (Na_2CO_3), L-ascorbic acid ($C_6H_8O_6$) and methanol (CH3OH), 3,5-Dinitrosalicylic acid (DNS acid), L-glucose ($C_6H_{12}O_6$), phenolphthalein ($C_{20}H_{14}O_4$), sodium sulfite (Na_2SO_3), sodium hydroxide (NaOH) and potassium sodium tartrate ($KNaC_4H_4O_6 \cdot 4H_2O$) all from Xilong (China).

2.1.3. Enzyme source

Enzymes used in this study included Bialfa-T, Glucozyme 2, and Biomatasa-L. These liquid form enzymes were produced by BIOCON company (India) and supplied by CTC Vietnam Nutrition Solution Limited Liability Company. These enzymes were stored at 5 - 7°C in the fridge. The attributes of the enzyme were displayed in Table 1.

2.2. Experimental design

2.2.1. The slurries preparation of brown rice and black bean

Brown rice (50 g) was roasted at 120°C in 45 min and 50 g of black beans were roasted at 150°C in 30 min in an air fryer (Paula Deen 9.5 Qt Family-Sized Air Fryer, USA). After that, the grain was added with 250 g of water and blended by a blender (Philips 600W blender, Netherlands) for 2 min. The slurry was used for subsequent enzyme hydrolysis treatments.

2.2.2. Effect of enzyme treatment on physicochemical properties of brown rice extract

For improving the recovery yield of the extracts of black bean and brown rice after hydrolyzing. The distribution company advises using enzyme combinations such as Bialfa-T - Glucozyme 2x or Biase - Biomatasa-L. Thus, the combined enzyme solutions were used in this study. Moreover, the effect of a single enzyme on the quality of the extracts of the black bean and brown rice was investigated to clarify the function of using mixed enzymes.

This experiment was also studied for the performance of physicochemical properties (i.e recovery yield, viscosity, and total soluble solids content) of the brown extracts under the hydrolysis activity, with fixed recommended conditions from the manufacturer and the result of the preliminary test. The experiment was triplicated.

Treatment 1. Bialfa-T enzyme treatment: The prepared slurry (prepare in section 2.2.1) was mixed well with 200 g water and heated to the temperature of 60°C using an electromagnetic

Table 1. The attribute of enzymes was used in this study*

Type	Activity	The hydrolysis conditions			Description
		pH	Temperature (°C)	Dose (g/100 g material)	
Bialfa-T	17500 IU/g	5.0 - 7.0	95 - 105	0.5 - 1.0	Bialfa-T is a liquid form and produced from *Bacillus Licheniformis*. It is an amylase enzyme (1.4-a-D-glucan-4-glucanohydrolase). The enzyme hydrolyses the glycoside alpha-d-1,4 bonds of the starch at random, producing soluble dextrins and oligosaccharides.
Glucozyme 2x	400 AGU/mL	4.0 - 5.5	65 - 70	0.25 - 0.5	Glucozyme 2x has a liquid form and produced from *Aspergillus Niger*. It is an exo-1,4-alpha-glucosidase (1,4D-Glucan glucanohydrolase). The enzyme can hydrolyze the alpha-D-1,6 branches as well as the alpha-D-1,4 polymeric bonds of the starch.
Biase	14000 IU/g	5.1 - 5.6	85 - 90	0.5 - 1	Biase has a liquid form and produced from non-GMO microorganisms. It has high beta-glucanase activity (endo-beta-1,3-1,4-glucanase). It acts on the amylose and amylopectin chains of the starch, turning them into short dextrin and maltose chains
Biomatasa-L	400 BioCon unit/mL	4.0 - 5.5	65 - 70	0.25 – 0.75	Biomatasa-L has a liquid form and produced from *Aspergillus Niger*. It has the ability to hydrolyse maltose in two glucoses. The final product is exclusively glucose

*: The information is supplied from the manufacturer.

stove (KGEB-1200, USA). The 0.1% Bialfa-T enzyme (g enyzme/g of grain) was added into the mixture (pH 6.2-6.5), stirred well, and incubated at 90°C for 30 min using a waterbath (WT-42, BioBase, China). Next, the mixture was then heated up to 100°C and kept for 10 min to inactivate the enzyme and filtered using cheesecloth to obtain the hydrolyzed extract. The extract was measured physicochemical properties in the same day.

Treatment 2. Glucozyme 2x enzyme treatment: The preparation for this treatment was done as previously described in section 2.2.1. The concentration of 0.05% Glucozyme enzyme and the incubation at 65°C for 60 min were applied for this treatment.

Treatment 3. The mixture of Bialfa-T and Glucozyme enzyme treatment: 100 g of water was added to the prepared slurry (section 2.2.1). The suspension was incubated at 90°C for 30 min to utilize the hydrolysis activity of the 0.1% Bialfa – T enzyme. The mixture was then added with 100 g of water and incubated at 65°C for 60 min for the hydrolysis of Glucozyme 2x, with a concentration of 0.05%. The activity of the enzymes in the mixture was inactivated at 100°C for 10 min before filtering using cheesecloth to obtain the hydrolyzed extract.

Treatment 4. Biase enzyme treatment: The preparation for this treatment was done as previously described in section 2.2.1. The concentration of 0.1% Biase enzyme and the incubation at 90°C for 30 min was applied for this treatment.

Treatment 5. Biomatasa-L enzyme treatment: The preparation for this treatment was done as previously described in section 2.2.1. Concentration of 0.05% Biomatasa enzyme and the incubation at 65°C for 60 min were applied for this treatment.

Treatment 6. The mixture of Biase and Biomatasa-L enzyme treatment: The preparation for this treatment was done as previously described in section 2.2.1. The suspension was incubated at 90°C for 30 min to utilize the hydrolysis activity of the 0.1% Bias enzyme. The mixture was then added with 100 g of water and incubated at 65°C for 60 min for the hydrolysis of 0.05% Biomatasa enzyme. The activity of the enzymes in the mixture was inactivated at 100°C for 10 min before filtering using cheesecloth to obtain the hydrolyzed extract.

Based on the results include recovery yield, viscosity, and total soluble solids of brown rice extract at each treatment. The best one was further investigated with the comparison on reducing sugar content, total phenolic content (TPC), and antioxidant capacity, to that of the control sample.

2.2.3. Effect of enzyme treatment on physicochemical of black bean extract

This experiment was done followed section 2.2.2; in which, the brown extract was replaced by black bean extract.

2.2.4. Effect of the ratio between brown rice extract to black bean extract on the sensory quality of product

The ratios between brown rice and black beans extracts investigated in this experiment were 1:2; 1:1; 2:1 (v/v). The grain extracts were prepared as expressed in sections 2.2.2 and 2.2.3. To make the solution, 1 L of the combined brown rice and black beans extracts were supplemented with sucrose ester of fatty acids (as emulsifier), pectin LMP, coconut milk powder, and sucrose with the concentrations of 0.003%, 0.7%, 2.0%, and 5% (w/v), respectively. The mixtures were homogenized at 15,000 rpm for 15 mins using a homogenizer (Ultra-Turrax® T 25, Germany) then heated to 80°C and hot poured into the glass bottle. The products were sterilized at 105°C for 10 min using a sterilizer (KT-40DP, Japan). The samples were stabilized for 24 h before sensorial evaluation.

2.3. Analytical method

2.3.1. Determination recovery yield

The recovery yield (%) of the extract was calculated as the following formula:

$$RY\ (\%) = \frac{A}{B} * 100$$

Where: RY is recovery yield (%). A is the mass of extract (g). B is the initial mass of sample (g).

2.3.2. Determination of viscosity

A viscometer (V-E Viscometer from AMETEK Brookfield, USA) was used to measure the viscosity of extracts at 30°C.

2.3.3. Determination of total soluble solids content

The total soluble solids content was measured by using a refractometer (Atago, 0-33%, Japan).

2.3.4. Extraction of bioactive compounds

The sample was extracted with 80% methanol at the rate of 1:9 (v/v). The mixture was stirred and left for 30 min at room temperature (29-31°C.). Next, the sample was filtered by filter paper (brand 102 qualitative) and diluted with 80% methanol (Xu et al., 2008).

2.3.5. Determination of total phenolic content

The method followed the procedure of Singleton & Rossi (1965) and Lim et al. (2007). The aliquot of 0.3 mL of the diluted extract was poured into test tubes, then 1.5 mL Folin-Ciocalteu reagent (diluted 10 times) and 1.2 mL of 7.5% sodium carbonate were added. The mixture was stirred well and left at room temperature for 30 minutes in dark. The absorbance was measured by using a spectrophotometer (Jasco V730, Japan) at 765 nm. The total phenolic content in this study were determined and calibrated with gallic acid ($y = 0.0133x + 0.0541$, $R^2 = 0.9982$). Its value was expressed as mg of gallic acid equivalent per 100 g dry matter (dm).

2.3.6. Determination antioxidant activity

The method followed the procedure of Thaipong et al. (2006). The aliquot of 0.2 mL of the diluted extract was mixed with 4 mL of 0.1 mM DPPH solution in a test tube. Then, the mixture was left 30 min at room temperature in the dark. The absorbance was measured by using a spectrophotometer (Jasco V730, Japan) at 517 nm. The antioxidant activity in this study were determined and calibrated with ascorbic acid ($y = -0.0098x + 1.1139$, $R^2 = 0{,}9985$). Its value was expressed as mg of ascorbic acid equivalent (AAE) per 100 g dry matter (dm).

2.3.7. Determination reducing sugar content

The method followed the procedure of Miller (1959). Preparation A solution: 10 g of 3,5-dinitrosalicylic acid (DNS acid), 2 g phenol, 0.5 g sodium sulfite; 10 g of sodium hydroxide, and 200 g sodium potassium tartrate were added into a 1000 mL volumetric flask and filled up to the mark by distilled water. The aliquot of 1 mL of the diluted extract was mixed with 3 mL of A solution in a test tube. Next, the test tube was heated in the boiling water for 5 min. After that, it was cooled quickly by an ice bath. The absorbance was measured by using a spectrophotometer (V-760 UV-Visible Spectrophotometer, USA) at 550 nm. The antioxidant activity in this study were determined and calibrated with glucose ($y = 0.0103x - 0.0983$, $R^2 = 0.9971$). Its value was expressed as mg of glucose equivalent (AAE) per 100 g dry matter (dm).

2.3.8. Determination sensory quality

The panelist panel includes 30 people who are students from 18 to 20 year olds. They did not use any food before 30 min of testing the sample. The participant received the 20 mL of the test sample was kept in a glass cup. The test samples were coded with a 3-digit number. The acceptability of consumers for the attribute of the sample such as color, odor, and the taste was evaluated based on the 7 points hedonic scale; in which 1: Strongly disliked and 7: Strongly liked. After testing each sample, the participant used water to clear the taste.

2.4. Statistical analysis

All experiments were done in triplicate. Results were expressed as mean ± standard deviation. The collected data were analyzed by using JMP 13.0 software and ANOVA One-way analysis of variance to determine the significant differences ($P < 0.05$). Diagrams were built by using the Microsoft Excel 2010 software.

3. Result and Discussion

3.1. Effect of enzyme treatment on physico-chemical properties of brown rice extract

3.1.1. Effect of enzyme treatment on recovery yield of brown rice extract

The different enzyme treatments significantly ($P < 0.05$) affected on the recovery yield, viscosity, and total soluble solids of the brown rice extract (Table 2). All of the samples treated with the mixed enzyme had a recovery yield (78 -

Table 2. Effect of enzyme treatment on physicochemical properties of brown rice extract

Treatments	Attribute		
	Recovery yield (%)	Viscosity (cP)	Total soluble solids (%)
Glucozyme 2x	$53.90^{e} \pm 3.05$	$63.67^{b} \pm 1.44$	$5.33^{d} \pm 0.29$
Bialfa-T	$64.16^{d} \pm 1.03$	$47.86^{d} \pm 0.79$	$8.17^{c} \pm 0.29$
Bialfa-T-Glucozyme 2x	$87.53^{a} \pm 1.52$	$27.70^{f} \pm 0.75$	$10.00^{a} \pm 0.50$
Biase	$67.83^{c} \pm 3.65$	$53.45^{c} \pm 0.45$	$7.83^{c} \pm 0.29$
Biomatasa-L	$51.24^{f} \pm 0.11$	$78.12^{a} \pm 0.14$	$3.83^{e} \pm 0.29$
Biase - Biomatasa	$78.33^{b} \pm 0.10$	$34.48^{e} \pm 0.38$	$9.00^{b} \pm 0.50$
P_{value}	< 0.0001	< 0.0001	< 0.0001

Values are expressed as mean ± standard deviation of three replications. The values have a different uppercase letter mean significant difference within the same column ($P < 0.05$) based on one-way ANOVA.

88%), total soluble solids (9-10%) higher than the single enzyme (51 - 71% and 3.8 - 8.0%, respectively). In contrast, brown rice extract's viscosity of the mixed enzyme was 27-34 cP and lower than the single enzyme (47 - 78 cP).

In between the four single enzyme treatments, the Biomatasa-L gave the hydrolysis extract had the highest viscosity (78.12 cP) as well as the lowest TSS (3.83%) and recovery yield (51.24%). While the brown rice extract treated with the Bialfa -T enzyme got the lowest viscosity (47.86 cP) and the highest TSS (8.17%) as well as the high recovery yield (64.16%). For the mix enzyme treatments, the Bialfa-T - Glucozyme 2x had greater activity than the Biase - Biomatasa in hydrolysis brown rice extract.

Moreover, the relation of the viscosity, recovery yield, and total soluble solid of the brown rice extract was found to fit the linear model (Figure 1). In detail, the viscosity of the extract decreased leading to the recovery yield and total soluble solids increased. The correlation coefficient of these attributes with the viscosity was $R^2 = 0.91$ and $R^2 = 0.93$, respectively.

The brown rice component has amylose and amylopectin (Abeysundara et al., 2017). It seems that during liquefaction and saccharification, the combination of Bialfa-T (cleaving the α - 1,4 glycosidic bonds present in the inner part of the amylose or amylopectin chains) and Glucozyme 2x (cleaving the α - 1,4 and 1,6 glycosidic bonds present in the inner part of the amylose or amylopectin chains) gave the hydrolysis activity higher than the single enzyme. On the other hand, using the endo enzyme (Bialfa-T) as the first enzyme in the hydrolysis period can help produce more amount of low molecular weight carbohydrates chain which promotes the hydrolysis ability of the exo-enzyme (Glucozyme 2x) as the second enzyme.

3.1.2. Effect of enzyme treatment on antioxidant activity of brown rice extract

The change of bioactive compound in brown rice extract under enzyme treatment also was found in this study (Table 3). As expected, the Biafla-T – Glucozyme 2x treatment improved the recovery yield, total phenolic content, and antioxidant activity of brown rice extract. These attributes increased approximately 2.4 times, 1.6 times, and 1.3 times, respectively. This phenomenon also occurred in previous studies such as the hydrolysis of brown rice with mesophilic α-amylase (Xu et al., 2015; Zeng et al., 2018). According to these authors, the starch in the cereal noticeably deteriorated which facilitated the extraction of the phenolic compounds from the food matrix during the hydrolysis period.

The results of viscosity, total soluble solids, and reducing sugar content as the evidence confirmed for the hydrolysis capacity of the enzyme Bialfa-T - Gluoczyme 2x in this experiment (Table 3). Specifically, the viscosity of brown rice extract decreased approximately 4.4 times, meanwhile, the total soluble solids and reducing sugar increased approximately 4 times and 21 times, respectively. This behavior agreed with the result of Konsula et al. (2004) and Rocha et al. (2010), who found the various starch such as rice, potato, corn, cassava after treating with α - amylase had the high reducing sugar content and low viscosity.

This phenomenon could be explained when being heated with water, the starch component in the extracted suspension absorbed water and swelled that making the extract became viscous. The extracted suspension of the control sample was with the absence of hydrolysis en-

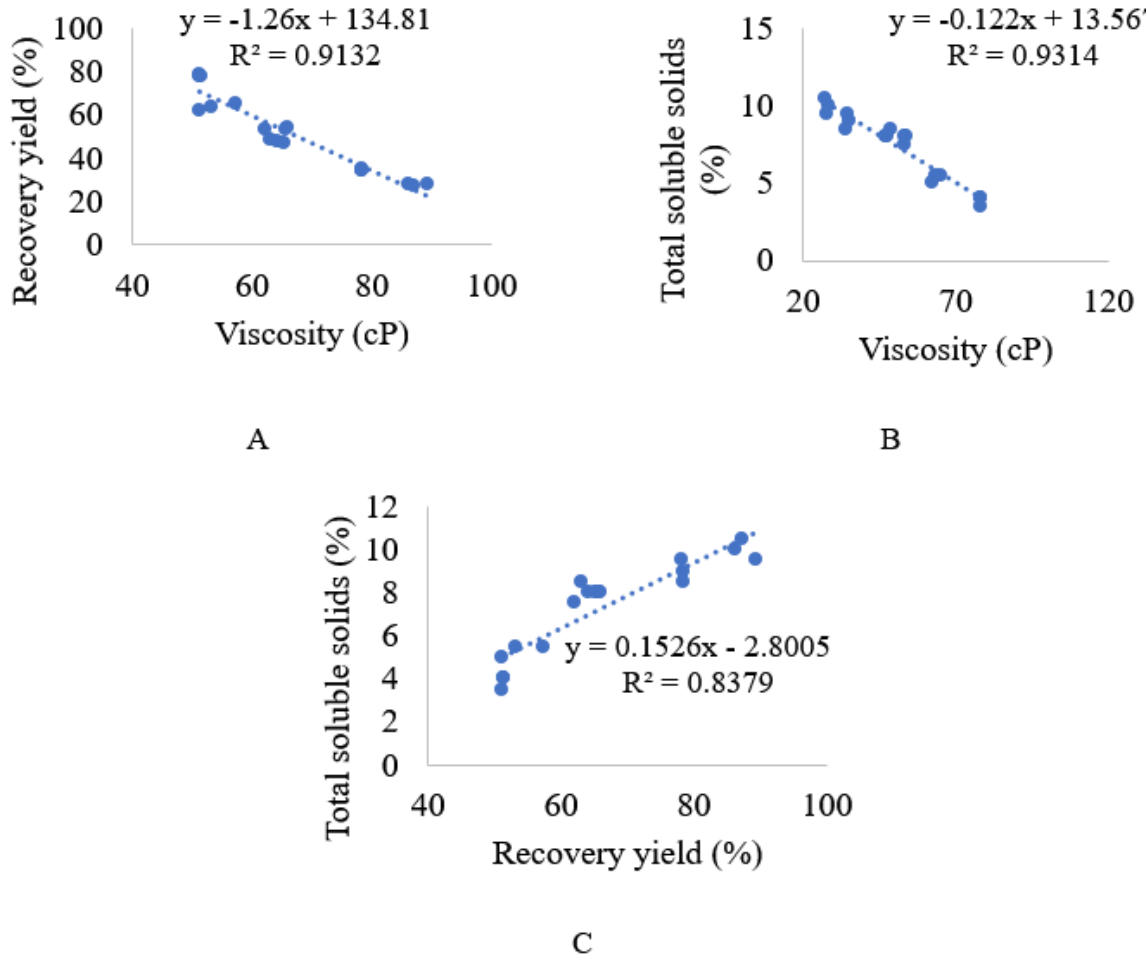

Figure 1. The correlation coefficient for the relationship between physicochemical properties of the brown rice extract (more specific what A, B, C)

zymes, the starch molecules had not been broken down into shorter polysaccharide chains and sugar molecules. That was the reason that caused the highest viscosity, hence the lowest recovery yield, compared to that of the other enzyme-treated samples.

Thus, the combination enzymes of Biafla-T and Glucozyme 2x was especially useful to improve the antioxidant activity, as compared to control sample.

3.2. Effect of enzyme treatment on physicochemical of black bean extract

3.2.1. Effect of enzyme treatment on recovery yield of black bean extract

According to the results in Table 4, the effect of different enzyme treatments on the recovery yield, viscosity, and total soluble solids of black beans extract was akin to what was shown on the brown rice extract. The black bean extract achieved the recovery yield (78.63 - 78.42%) and TSS (6.00%) under the mix enzyme treatment higher than the single enzyme treatment (43.12 - 70.10%, 3.67 - 4.83%, repestively). Correspondingly these treatments, the black bean extract got the lowest viscosity of range 46.10 - 52.50 cP and the highest viscosity of range 52.50 - 81.99 cP; respectively.

In between the Bialfa-T - Glucozyme and Bias - Biomassa treatments, there is no statistically significant difference to recovery yield and TSS. However, the recovery yield's mean value of the Bialfa-T - Glucozyme treatment was still higher than Bias - Biomassa treatment. In addition, both the recovery yield and total soluble solids in black bean extract had a negative relative with

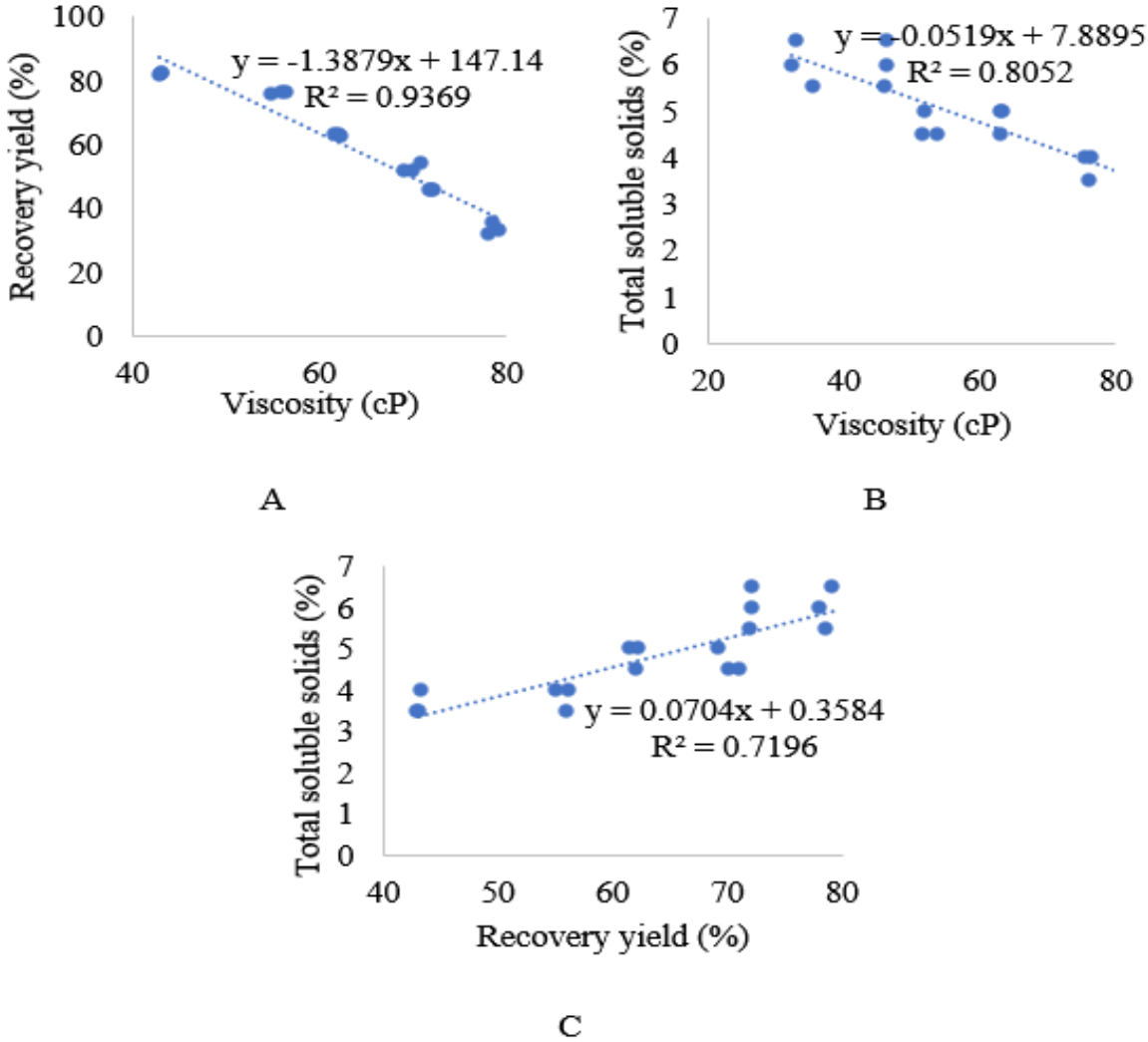

Figure 2. The correlation coefficient for the relationship between physicochemical properties of the black bean extract.

the viscosity; which reached the correlation coefficients were $R^2 = 0.93$ and $R^2 = 0.80$; however, the total soluble and recovery yield had a positive relative ($R^2 = 0.72$) (Figure 2).

3.2.2. Effect of enzyme treatment on antioxidant activity of brown rice extract

The enzyme treatment was useful to increase the recovery yield of black bean extract. For example, this parameter of a sample with enzyme and without enzyme treatments is 79.62% and 21.41%, respectively (Table 5). The same behaviour as what shown on brown rice extracts, the combined Bialfa-T and Glucozyme 2x enzymes treatment released more bioactive compound from the black bean into the extract. In detail, the solution had higher total phenolic content (10.93 mg GAE/100 g dm), antioxidant activity (7.15 mg AAE/100 g dm), and the reducing sugar content (81.94 mg GE/100 g dm) than the enzyme untreated samples (8.94 mg GAE/100 g dm, 4.01 mg AAE/100 g dm, and 81.94 mg GE/100 g dm, respectively). The change of physicochemical of the black bean extract under the enzyme treatment could be explained similarly the brown rice.

3.3. Effect of ratio between brown rice extract to black bean extract on the sensory quality of product

Table 6 presents the sensorial quality of the product with different mixing ratios between

Table 4. Effect of enzyme treatment on physicochemical properties of black bean extract

Treatments	Attribute		
	Recovery yield (%)	Viscosity (cP)	Total soluble solids (%)
Glucozyme 2x	$43.12^{e} \pm 0.15$	$75.99^{b} \pm 0.37$	3.83c ± 0.29
Bialfa-T	$61.97^{c} \pm 0.39$	$52.50^{d} \pm 1.20$	$4.67^{b} \pm 0.29$
Bialfa-T - Glucozyme	$78.63^{a} \pm 0.54$	33.55f ± 1.72	$6.00^{a} \pm 0.50$
Biase	$70.10^{b} \pm 0.90$	$63.12^{c} \pm 0.14$	$4.83^{b} \pm 0.29$
Biomatasa-L	$55.76^{d} \pm 0.68$	$81.99^{a} \pm 0.14$	$3.67^{c} \pm 0.29$
Biase - Biomatasa	$78.42^{a} \pm 0.63$	$46.10^{e} \pm 0.15$	$6.00^{a} \pm 0.50$
P_{value}	< 0.0001	< 0.0001	< 0.0001

Values are expressed as mean ± standard deviation of three replications. The values have a different uppercase letter mean significant difference within the same column ($P < 0.05$) based on one-way ANOVA.

Table 3. Physicochemical properties of the brown rice extract untreated and treated with enzyme

Treatments	Recovery yield (%)	Viscosity (cP)	Total soluble solids (%)	TPC (mgGAE/100g dm)	Antioxidant activity DPPH (mg AAE/100 g dm)	Reducing sugar (mg GE/100 g dm)
The control sample	$36.45^{b} \pm 3.09$	$118.61^{ba} \pm 1.68$	$2.50^{b} \pm 0.50$	$1.09^{b} \pm 0.03$	$1.33^{b} \pm 0.03$	$8.81^{b} \pm 0.15$
Bialfa-T - Glucozyme 2x	$88.54^{a} \pm 1.57$	$27.70^{b} \pm 0.75$	$10.33^{a} \pm 0.76$	$1.69^{a} \pm 0.12$	$1.74^{a} \pm 0.07$	$193.37^{a} \pm 1.08$
P_{value}	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001

Values are expressed as mean ± standard deviation of three replications. The values have a different uppercase letter mean significant difference within the same column ($P > 0.05$) based on one-way ANOVA.

Table 5. Physicochemical properties of the black bean extract untreated and treated with enzyme

Treatments	Recovery yield (%)	Viscosity (cP)	Total soluble solids (%)	TPC (mgGAE/100g dm)	Antioxidant activity DPPH (mg AAE/100 g dm)	Reducing sugar (mg GE/100 g dm)
The control sample	$21.41^{b} \pm 0.55$	$88.05^{a} \pm 0.13$	$1.83^{b} \pm 0.24$	$8.94^{b} \pm 0.07$	$4.01^{b} \pm 0.20$	$4.17^{b} \pm 0.03$
Bialfa-T - Glucozyme	$79.62^{a} \pm 1.54$	$33.55^{b} \pm 0.17$	$6.50^{a} \pm 0.24$	$10.93^{a} \pm 0.07$	$7.15^{a} \pm 0.17$	$81.94^{a} \pm 1.48$
P_{value}	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001

Values are expressed as mean ± standard deviation of three replications.
The value have a different uppercase letter mean significant difference within the same column ($P > 0.05$) based on one-way ANOVA.

Table 6. Sensory quality of the product under the different mixing ratio between brown rice and black bean extracts

Brown rice and black bean extracts ratio (v/v)	Attribute		
	Color	Odor	Taste
1 : 2	$4.27^{c} \pm 0.58$	$4.37^{c} \pm 0.49$	$3.83^{c} \pm 0.79$
1 : 1	$5.27^{b} \pm 0.52$	$6.43^{a} \pm 0.86$	$6.23^{a} \pm 0.94$
2 : 1	$6.03^{a} \pm 0.61$	$4.90^{b} \pm 0.96$	$5.47^{b} \pm 1.01$

1: Strongly disliked and 7: Strongly liked

brown rice and black beans extracts. The perceptions of panelists on the flavor of the product fluctuated, the total highest value for these attributes (5.27 - 6.43) was obtained on sample 1:1, and the lowest (3.83 - 4.27) was with the sample 1:2. The reason could be explained based on the balanced and harmonious flavor between the two ingredients.

4. Conclusions

The combination of Bialfa-T and Glucozyme enzyme resulted in the best effect on physicochemical properties and bioactive compounds for both brown rice and black bean extracts. In particular, the enzyme treatment gives the recovery yield and the antioxidant capacity of the brown rice extract higher 2.4 times and 1.3 times, respectively, than the control sample. Similarly, the hydrolyzed black bean extract has the recovery yield and the antioxidant capacity higher 3.7 times and 1.8 times, respectively than the control sample. The plant milk recipe has the ratio between brown rice extract and black bean extract 1:1 (v/v) to get the best sensory.

Conflict of interest

The authors declare no conflict of interest.

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Starch recovery from turmeric powder (*Curcuma longa*) after ethanol curcumin extraction in comparison to the conventional method

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ABSTRACT

Recovery starch from organic waste significantly contributes to sustainable agricultural production. This study aimed to recover starch from the waste generated from the curcumin extraction by using ethanol. The physicochemical properties of the isolated starch such as microscopic morphology, Fourier transform infrared spectroscopy, X-ray diffraction, total starch, iodine binding capacity of starch, curcumin content determined by high-performance liquid chromatography were compared to that of starch obtained from the conventional method of extraction from the fresh rhizome. The results showed that the starch obtained from the fresh rhizome had a higher yield compared to that of starch isolated from the turmeric powder after extracting curcumin (21.3% vs. 8.5%). The total starch analysis indicated that the former starch had a higher purity (98% vs. 77%, dw). The SEM imaging showed that both starches had irregular shapes with a thick flat and smooth surface. Although the starch isolated from the turmeric powder showed the dedicated properties of starch, the peak intensity and crystalline structure were remarkably decreased, via FTIR and X-ray diffraction analyses, respectively. The pasting analysis showed a clear change in starch obtained from the turmeric powder after ethanol extracting curcumin since a low peak viscosity was recorded. The HPLC curcumin quantification showed that both starches had a very low residue of curcumin (18.4 mg/100 g and 66.5 mg/100 g, dw). The process of starch recovery after curcumin extraction from turmeric would be further improved to prevent the changes in physicochemical properties and for better yield.

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1. Introduction

Tumeric (*Curcuma longa*) is commonly distributed in tropical and sub-tropical regions. In Vietnam, turmeric is widely cultivated in northern and highland areas. One of the most significant components of the rhizome is the curcuminoids that have been intensively investigated. Curcuminoids from turmeric have been reported as a natural food colorant, preservative, and *in vitro* anticancer (Yu & Huang, 2010), antioxidants and inflammatory agents (Anand et al., 2008).

Starch is one of the major components in the turmeric rhizome. Starch accounts for 47% of the dried weight of the rhizomes (Leonel et al., 2003). In another study, the turmeric rhizome contained approximately 67% starch (dw) (Kuttigounder et al., 2011). Regardless of its high starch content, the application has been seldom extended in the food and pharmaceutical industries. According to Santana et al. (2017), turmeric starch is essential as an alternative starch source for the food industry. Although several researchers

have been addressed on isolation and characterization of the physicochemical properties of starch from fresh turmeric rhizome (Jyothi et al., 2003; Kuttigounder et al., 2011; Sajitha & Sasikumar, 2015), the recovery starch after curcuminoids extraction seem very limited. It has been reported that turmeric starch was successfully recovered from supercritical fluid and would be essential use for dietary starch (Santana et al., 2017).

Ethanol extraction of curcuminoids has been utilized as a green method of solvent extraction from the turmeric powder (Osorio-Tobón et al., 2016; Lateh et al., 2019; Patil et al., 2019). However, starch recovery from the residue of ethanol curcumin extraction has not been investigated yet. The present study aimed to isolate starch from turmeric powder after curcumin extraction by using ethanol. The physicochemical properties of the isolated starch were then analyzed and compared to those of the starch obtained from the conventional method of extraction from the fresh turmeric by precipitation to evaluate its potential application in the industry.

2. Materials and Methods

Materials: the mature (at least one-year-old) yellow turmeric rhizomes (*Curcuma longa*) were procured from Ea Bhok, Cu Kuin district, Dak Lak province, Viet Nam.

Chemicals: Curcumin (Himedia, India), acetonitrile, orthophosphoric acid, ethanol (HPLC grade, Merck) were obtained from the local supplier. Hydrochloric acid (37%), iodine, sodium hydroxide pellet, dimethyl sulfoxide (analytical graded) were purchased from a local supplier.

2.1. Isolation of starches

2.1.1. Isolation turmeric starch from the fresh rhizome

The procedure of isolating starch from fresh turmeric rhizome followed the procedure developed by Nakkala et al. (2020) with modifications. The rhizomes were cleaned and sliced into slices (around 2 mm thick) using a domestic slicer. The turmeric slices were then immediately soaked and ground in sodium metabisulfite 0.02% (w/v) (the ratio of turmeric and sodium metabisulfite was 1:4). After grinding, the turmeric was soaked in the sodium metabisulfite for further 12 hours. The finely ground sample was then filtered through a cheesecloth to collect the solution containing starch. The retained portion was re-slurried with 10 L water and filtered for the second time. The solution collected from the second filtration was pooled with the first solution. The starch was precipitated overnight without disruption. The clear white-yellowish layer of turmeric starch was decanted and dried in a heat pump dryer at a temperature of 35°C until reached the moisture content of approximately 10%. The dried starch was passed through a 300 µM sieve and the starch powder was stored in a sealed aluminum bag at room temperature for further analysis.

2.1.2. Isolation of starch from turmeric powder after ethanol curcumin extraction

To mimic the starch recovery from waste generated from the curcumin extraction process, the turmeric rhizomes were initially curcumin extracted. The slices of turmeric rhizome (similarly prepared as aforementioned) were dried in a heat pump dryer at 35°C until reached the moisture content around 10%. The dried turmeric slices were then pulverized into powder by using a domestic grinder. To avoid overheating, the grinder was intermittently stopped for 30 sec for every 30 sec-grinding process. The ground sample was passed through a 300 µM sieve. The fine turmeric powder was extracted with absolute ethanol with the ratio of 1:100 at 50°C for 8 h to mimic the curcumin extraction process. After the extraction process, the sample was filtered through a filter paper and the residue was collected for starch extraction. The sample was re-slurried into 0.02% (w/v) sodium metabisulfite with the ratio of 1:4 and soaked overnight. The slurry was filtered through a cheesecloth. The retained samples were then mixed with 0.02% (w/v) sodium metabisulfite and refiltered. The starch solution was combined and the starch was precipitated, dried at 35°C, passed through a sieve again as previous procedure. The starch powder was put in an aluminum bag, sealed, and stored at room temperature for further analysis.

2.2. Determine total starch

The total starch was estimated based on the acid hydrolysis method (Kent-Jones & Amos, 1960) with modifications. Isolated starches of 2.5 g were hydrolyzed in 220 mL of 3.36% (v/v) hy-

drochloric acid solution in a flask. The starch mixture was heated to 90°C for 2.5 h and then cooled before being neutralized with NaOH 5 N. The volume of the acid hydrolysate was adjusted to the final volume of 250 mL using deionized water. The sugar content in the solution was determined by the colorimetric method using DNS reagent as suggested by (Başkan et al., 2016).

2.3. Iodine binding capacity

The iodine binding capacity of isolated starches was evaluated following the method described by Peng & Perlin (1987). Starch (50 mg) was dispersed in 5 mL dimethyl sulfoxide (DMSO) by heating in a 100 mL volumetric flask to obtain a clear slurry. Then the volume was adjusted to 100 mL. Two milliliters of the diluted starch slurry were transferred into a second volumetric flask (50 mL) in which 1 mL of NaCl 1 M, 40 mL of water, and 1 mL of iodine solution (containing a mixture of 2 mg I_2 and 20 mg of KI). The final volume was brought to 50 mL. The color was developed in 30 min and then the absorbance at 600 nm was measured by using a spectrophotometer and a 1 cm cuvette. The blue value (BV) was calculated based on the recommended equation: BV = (4 x A_{600})/C where A_{600} is the absorbance at 600 nm and C is the concentration of the starch (mg/L) in the solution.

2.4. Scanning electron microscopy (SEM)

The isolated starches were spread on a metal stub attached with a double-sided adhesive carbon tape. The samples were coated with a thin layer of Pt. The imaging was acquired by using FE-SEM S4800 (Hitachi, Japan). The coated samples were imaged in a scanning electron microscope (SEM), Hitachi S-3400 (Tokyo, Japan) at an accelerating voltage of 10 kV and a working distance of 8.0 mm.

2.5. Pasting properties

The pasting properties of the isolated starchers were investigated using Brookfield Engineering Labs (DV2T) system followed the procedure of Rapid Visco Analyzer (RVA) with modifications. The starch solution (7%, w/v) was prepared in water, mixed and then subjected to the system for measurement with the protocol as follows: the spindle speed was kept at 160 rpm. The length of the analysis was 12.5 min. The temperature was kept at 55°C from the first 2 min, followed by an increase from 55 to 90°C in the next 3 min. From 5 - 9 min, the temperatures remained at 90°C before cooling down to 55°C at 12.5 min. The pasting profile was acquired and the viscosity was presented in centipoise (cP).

2.6. Fourier transform infrared analysis

The short-range order structure of starches was examined based on the technique of Fourier transform infrared (PerkinElmer MIR/NIR Frontier). The sample was prepared with KBr and the FTIR spectra were recorded in the range of 4000 cm^{-1} to 400 cm^{-1} with a resolution of 4 cm^{-1} per scan.

2.7. X-ray diffraction analysis

The crystalline pattern of isolated starches was analyzed by X-ray diffraction (Bruker D2 Phaser, Germany). The instrument was equipped with a copper X-ray generator working in conditions of 40 kV and 80 mA. 2θ range of 4–60° was used to acquire for X-ray diffractograms with a step size 2θ of 2.0°/min.

2.8. Curcumin analysis by high-performance liquid chromatography

Curcumin in turmeric powder and starches were quantified by using high-performance liquid chromatography (HPLC) following the suggested procedure from Moorthi et al. (2013) with modifications. Sample (50 mg) was extracted in 20 mL ethanol 60% (v/v) in a 50 mL centrifuge tube. The tube was then vortexed for 30 sec for every 15 min in total 2 h. The sample was then centrifuged (4000 rpm, in 10 min) to obtain a clear supernatant. The clear supernatant was filtered through a PTFE (0.45 µM) membrane and transferred into an amber HPLC vial for analysis. HPLC system (Shimadzu, Japan) was equipped with LC20-AD pump, CBM-20Alite controller and PDA detector. C18 column (Inertsil-ODS 3, 250 x 4.6 mm, 5 µM) was used as stationary phase. The mobile phase was a mixture of orthophosphoric acid (0.1%) and acetonitrile (45:55, v/v) with the isocratic flow at 1 mL/min. The curcumin was detected at 427 nm. Curcumin standard (Himedia, India), was used to generate the calibration curve for quantification of curcumin in the samples.

3. Results and Discussion

3.1. Yield and total starch of the isolated starches

The yield of isolated starches were presented in Figure 1. The yield of starch isolated from the fresh turmeric rhizomes was around 21.3% (dw). This yield was significantly higher ($P < 0.01$) compared to that of starch isolated from the turmeric powder after extracting curcumin by ethanol (21.3% vs. 8.5%, Figure 1A). The low recovery of starch from the turmeric powder after ethanol extraction was possibly due to during the drying process of turmeric powder preparation, the starch was entrapped within the cellulosic pockets. Moreschi et al. (2006) observed the turmeric rhizome under the SEM reveal that starches allocate within the cellulosic pocket. Thus, in this study, although the turmeric powder was ground into approximately 300 µM particles, the starch seems still to be kept in the cell wall and limited the escape during starch extraction. The yield obtained from the fresh rhizomes is comparative to that reported in a study by Nakkala et al. (2020). However, in general, the starch yield was lower than that recorded in other studies in which the yield ranged from 40-60% (Leonel et al., 2003; Kuttigounder et al., 2011; Sajitha & Sasikumar, 2015).

The results of total starches are presented in Figure 1B. The starch isolated from the fresh rhizome had a higher total starch content compared to that collected from the turmeric powder after extracting curcumin with ethanol (98.3% vs. 77.2%, dw). The finding suggests that the starch isolated had a better purity compared to that of starch obtained from the turmeric powder. This result agreed with the previous report since the starch has been recovered from the fresh rhizome has a high purity (Alcázar-Alay & Meireles, 2015). The starch isolated from the fresh turmeric rhizome, however, had a higher total starch content reported earlier (ranges from 77-87%) (Leonel et al., 2003; Braga et al., 2006). The isolation of starch from the turmeric powder after extracting curcumin was in an attempt to recover starch from organic waste generated from the industry of curcumin extraction. The low yield and total starch of this starch suggest an improved method for starch recovery.

3.2. Iodine binding capacity of isolated starches

The iodine binding capacity of starch mostly involves the complex of amylose and iodine. The iodine capacity may act as an index for the apparent amylose content of the starch. For the native starch, particularly after isolation, the amylose leaching would be limited and the starch has a high blue value. The blue value of the starch isolated from the fresh rhizome was significantly ($P < 0.01$) higher than in the starch isolated from the turmeric extraction residue (0.589 vs. 0.123) (Table 1). The blue value of starch obtained from fresh turmeric was slightly higher than reported in the literature (blue value of 0.427 in Pham & Vo (2017)). A low blue value was observed in the starch isolated from the turmeric powder that would be involved in the process of extraction. In this study, the turmeric powder was extracted with ethanol for 8 h at 50°C. At these extraction conditions, the starch in the powder appears to be annealed resulting in changes of physicochemical properties, as an example, reduced in the blue value. Lan et al. (2008) reported that the annealing process of starch occurred when starch was treated under the gelatinization temperature of the starch. The annealed starch reduces in iodine binding capacity due to rearrangement at the molecular level, particularly the amylose molecule.

Table 1. The blue value of starches isolated from the fresh rhizome and turmeric powder

Starch	Blue Value
Isolated from the fresh turmeric rhizome	0.589 ± 0.03^{a}
Isolated from the turmeric powder after curcumin extraction	0.123 ± 0.01^{b}

Values are expressed as mean ± SD of triplicates.
Values followed by different lowercase letters in superscripts were significantly different at $P < 0.01$.

3.3. Scanning electron microscopic imaging (SEM)

The morphology of the starch was observed under the SEM and the images are presented in Figure 2. The turmeric starch had an irregular, nearly oval and flat shape. The starches had a smooth surface and were around 5 µM thick. The starches granules can be classified into the small

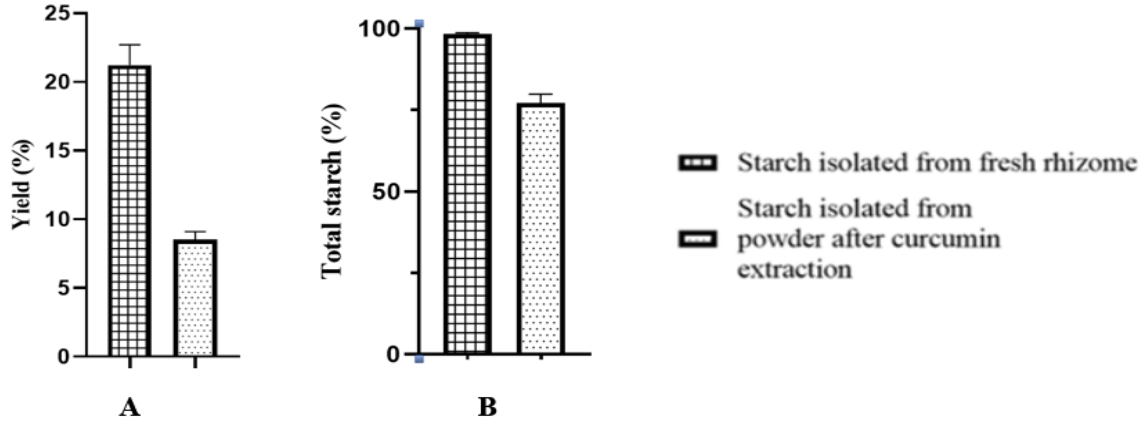

Figure 1. The recovery yield (A) and total starch (B) of starches isolated from the fresh rhizome and from the turmeric extraction residue.

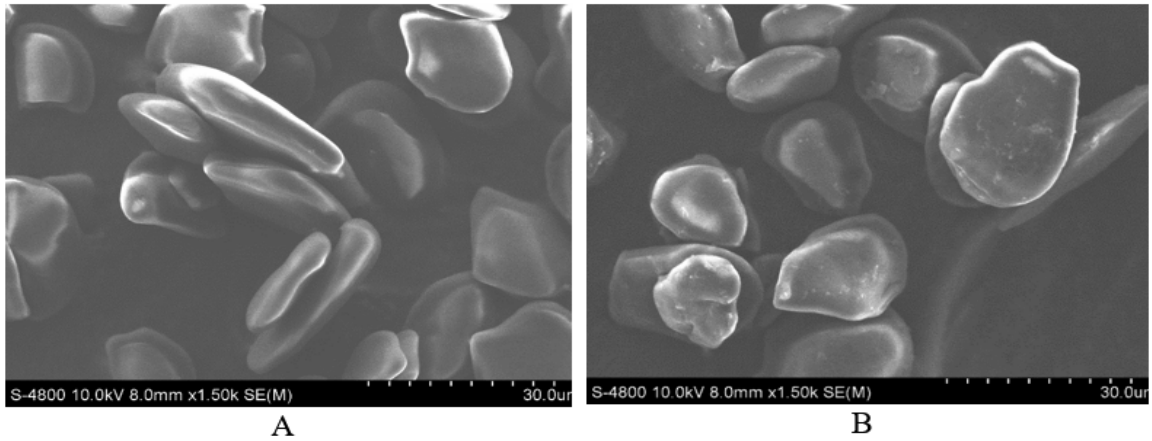

Figure 2. SEM imaging of the Curcuma starches isolated from the fresh rhizome (A) and isolated from the turmeric powder after curcumin extraction, the SEM acquired at the magnification of 1500x.

granule (< 20 µM long) and large (> 20 µM long). The morphology of the starch was consistent with descriptions in earlier studies (Jyothi et al., 2003; Leonel et al., 2003; Pham & Vo, 2017). The starch isolated from the fresh rhizome had a clear, clean surface (Figure 2A) whereas the stained surface (Figure 2B) was observed in the starch isolated from the powder after curcumin extraction. The SEM imaging confirms the difference in purities of starches found in this study.

3.4. Pasting profile of isolated starches

The pasting properties of starches were analyzed and the profiles are presented in Figure 3. The pasting profile of starch isolated from the fresh rhizome was characterized by the pasting properties of a native starch (Figure 3A). The pasting temperature of this starch was 78.3oC and the peak viscosity was reached around 732 cP. The starch paste showed resistance in shear stress since no breakdown was observed during applying the shear rate of 160 rpm at 90°C. When the starch paste was cooled down to 55°C, the viscosity rapidly increased and reached the final viscosity of 1037 cP. This profile of this starch was quite similar to that reported in the literature. Pham & Vo (2017) found that during an analysis of viscosity of Curcuma starch paste, the breakdown was not observed and the final viscosity was also relatively high. A significant change in the pasting profile was recorded in starch isolated from the turmeric powder after curcumin extraction (Figure 3B). Although the pasting temperature negligibly (around 80°C), the peak viscosity was very low (around 106 cP). The low shear stress resistance was recorded since the viscosity was remarkably reduced. The set-

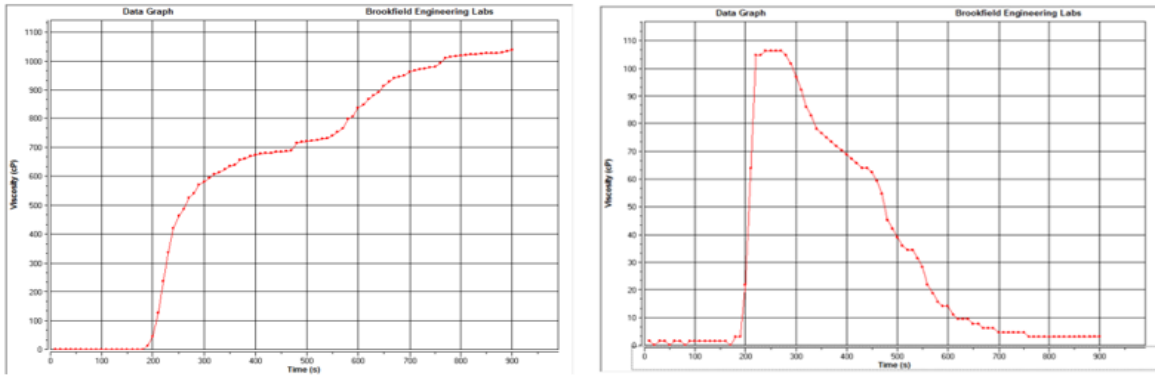

Figure 3. The pasting profiles of starches isolated from the fresh rhizome (A) and the turmeric powder eliminated curcumin (B), measured by Brookfield Engineering Labs viscometer.

back in viscosity during cooling was also not observed. These pasting characteristics indicate that the starch was modified toward annealing as previously mentioned. The annealed starches can keep the pasting temperature but greatly decrease swelling power and final viscosity (Lan et al., 2008; Jayakody et al., 2009).

3.5. Fourier transform infrared spectroscopy (FTIR)

The FTIR spectra of starches (Figure 4) showed that both starches had the dedicated peaks of starch in the regions of 3000 - 2900 cm^{-1}, 1150 - 1100 cm^{-1} and 1100 - 900 cm^{-1}. The FTIR spectrum peak intensity of starch isolated from the curcumin powder was noticeably reduced compared to that of starch isolated from the fresh rhizome. This finding again confirms for physicochemical change of starch in the powder undergone the extraction of curcumin by using ethanol for 8 h at 50°C. The treatment conditions possibly caused a partial modification of the starch that led to a reduction in the FTIR peak intensities of starch absorptive bands. Xu et al. (2021) observed the partial modification of potato starch in moist-heat treatment at 60°C in 30 mins indicates the absorption at the bands of 1047 cm^{-1} and 995 cm^{-1} were greatly decreased. In this study, the absorbance at 995 cm^{-1} of starch isolated from the curcumin powder after eliminating curcumin reduce from 70.5% to 67.4% compared to that of starch isolated from fresh turmeric. A similar trend was also observed in the absorbance at the band of 1047 cm^{-1} (74.3% to 68.1%). The absorbance at 995 cm^{-1} is considered as bending of C-OH boning, which is responsible for hydroxyl groups of starch whereas the absorbance at 1047 cm^{-1} represents the order structure of the starch (Warren et al., 2016; Xu et al., 2021).

3.6. X-ray diffraction analysis

X-ray diffraction analysis was performed to clarify the long-range structure of isolated starches and the results are presented in Figure 5. The Curcuma starches had a clear B-type crystalline pattern that is dedicated to the starch originating from root or rhizome. The results agreed with previous studies in which the B-type crystalline was found in turmeric starch (Kuttigounder et al., 2011; Pham & Vo, 2017). Although different in the peak intensity, the peaks were well pronounced at 6.3, 9.6, 11.9, 14.5, 17.1 and 18.7 Å in two obtained starches. The peak at 11.9 Å had the highest intensity followed by a peak at 17.1 and 18.7 Å. The finding in this study is different from what was reported in a study by Kuttigounder et al. (2011) in which the peak at 17 Å was recorded with the highest intensity. The difference in the most pronounced peak would be due to the difference in origin of starches. The relative crystallinities of starches isolated from the fresh rhizome and the turmeric powder after extracting curcumin were 26.8 and 20.4%, respectively. The crystallinity of starches obtained from this study was in the reported range of 15 to 45% (Zobel, 1988). The starch isolated from the turmeric powder after curcumin extracting, as aforementioned, was partially modified and

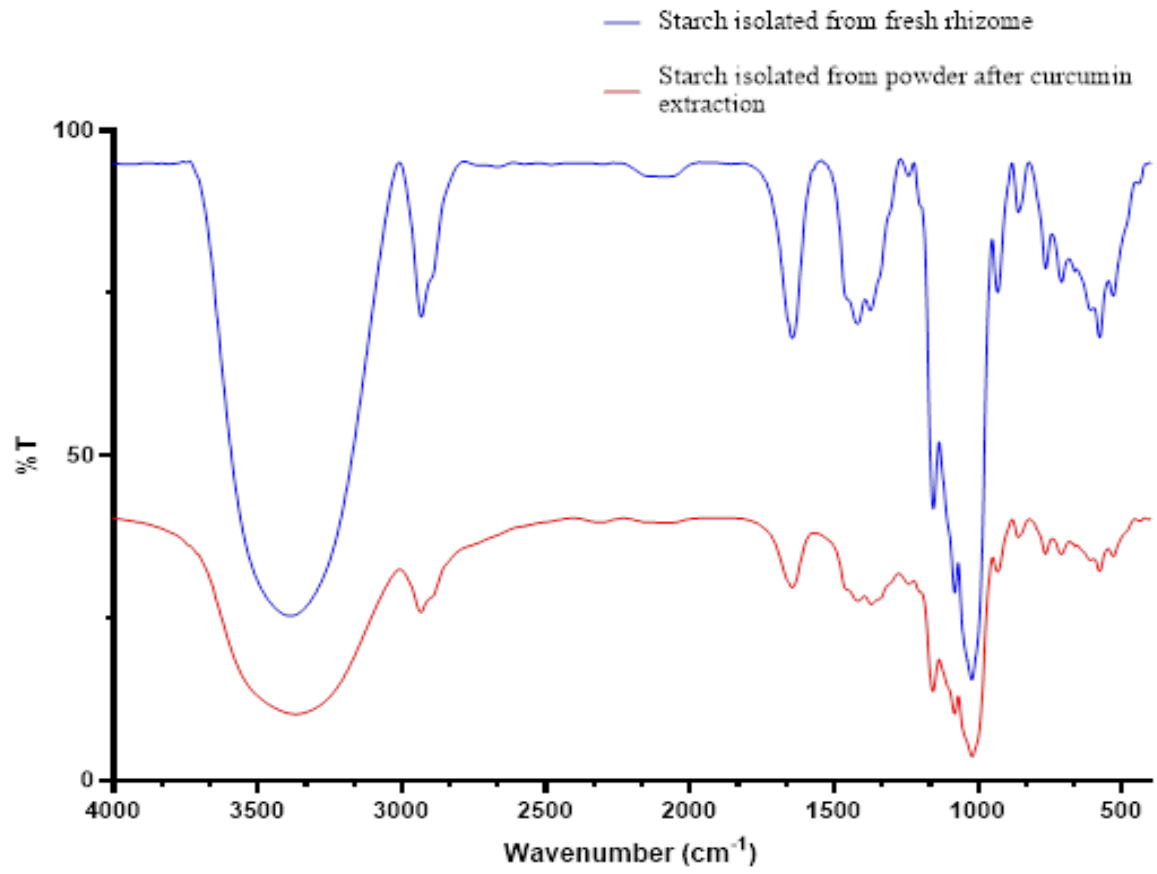

Figure 4. FTIR spectra of starches isolated from the fresh rhizome and the turmeric powder after extracting curcumin.

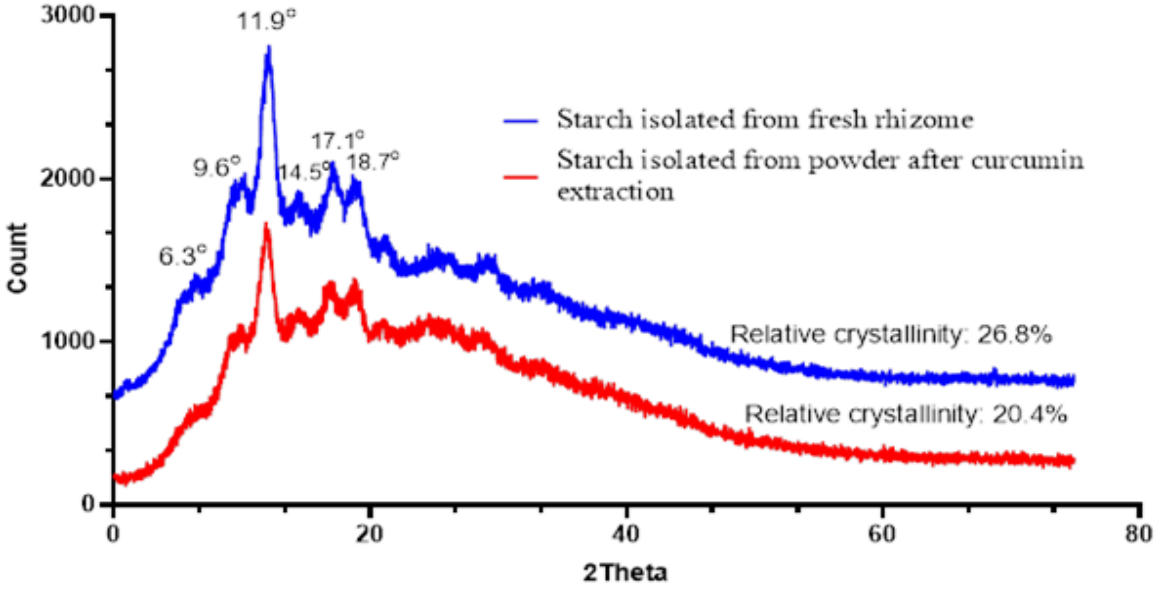

Figure 5. X-ray diffractograms of isolated starches from two different methods, data were offset for clarity.

thus likely led to a reduction in the relative crystallinity.

3.7. Curcumin analysis

The curcumin in turmeric powder and starches were quantified by using RP-HPLC-PDA method. The chromatograms and the curcumin results are presented in Figure 6 and Table 2, respectively. Previous studies demonstrate that curcumin exists in turmeric with other curcuminoids. The RP-HPLC-PDA elution of curcuminoids was in order as bisdimethoxy curcumin, bisdimethoxy curcumin and curcumin (Gugulothu et al., 2012; Peram et al., 2017). In the present study, bisdimethoxy curcumin, dimethoxy curcumin and curcumin were orderly eluted at 10, 10.84 and 11.756 min (Figure 6). The curcumin was predominant over other curcuminoids with the total peak area accounting for over 85%. Quantitative analysis showed that the powder contained 3.746 mg/100 g (dw) curcumin. The level of curcumin in turmeric (Curcuma longa) was higher than the level documented. Two varieties of turmeric in Vietnam analyzed before contained from 2.977 to 3.198 mg/100 g (Hayakawa et al., 2011). According to this study, the level of curcumin from Vietnamese turmeric was higher than that of varieties collected from Thailand, Japan, and Indonesia. The residues of curcumin in the isolated starches were very low ranged from 18.4 to 66.5 mg/100 g (dw) (Table 2). The starch isolated from the conventional method of using fresh turmeric had a very low curcumin level (18.4 mg/100 g), indicating the limited application based on the biological curcumin in the starch. The curcumin remained in the starch isolated after ethanol extracting was 66.5 mg/100 g, suggest for the effectiveness of using ethanol to extract the curcumin since over 98% of curcumin was extracted.

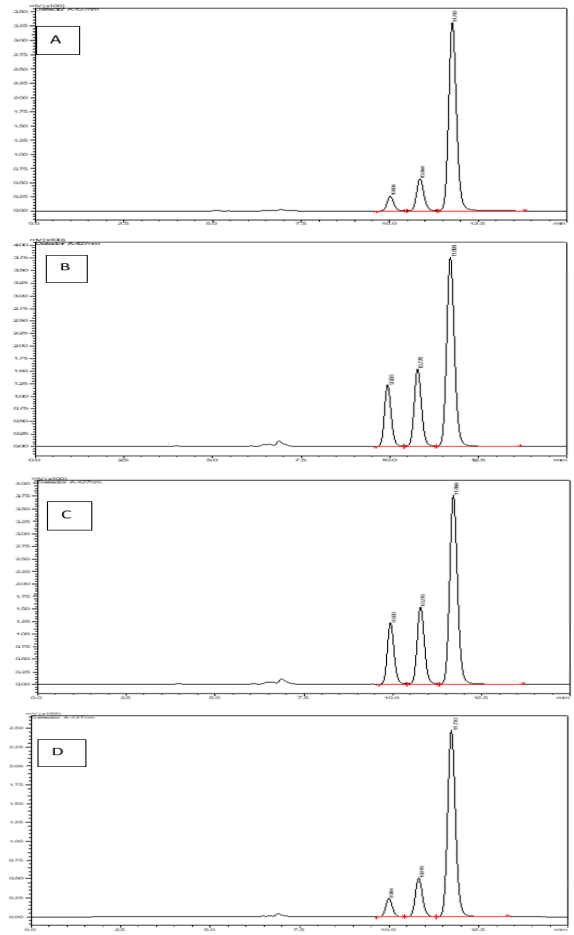

Figure 6. Chromatograms of (A) curcumin standard, (B) in turmeric powder, (C) in starch isolated from the fresh rhizome, and (D) in starch isolated from the turmeric powder after eliminating curcumin by ethanols, the analysis using RP-HPLC-PDA.

Table 2. The blue value of starches isolated from the fresh rhizome and turmeric powder

Sample	Curcumin content (mg/100 g)
Turmeric powder	3,746.5 ± 141.5[a]
Starch isolated from fresh rhizome	18.4 ± 0.9[b]
Starch isolated from the turmeric powder after curcumin extraction	66.5 ± 3.6[c]

Values are expressed as mean ± SD of triplicates.
Values followed by different lowercase letters in superscripts were significantly different at $P < 0.05$.

4. Conclusions

The starch was initially recovered from the turmeric powder after curcumin extraction by using ethanol for 8 h at 50°C. The obtained starch had a moderate yield of extraction and purity. The physicochemical properties such as iodine binding capacity, SEM imaging, pasting properties, FTIR and X-ray diffraction were analyzed. The results showed that the starch was partially modified. Quantitative analysis proved that ethanol extraction effectively removed curcumin from the starch powder, only around 0.5% curcumin residue in the isolated starch. Modifications should be implemented to improve the pro-

cedure of starch recovery from the organic waste originating from the curcumin extraction process.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

The authors declare no conflicts of interest.

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